Identification and Characterization of a Cancer Initiating Subpopulation in Pediatric Sarcomas

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1. Summary

Cancer stem cells (CSCs) have been identified from a number of solid tumors, but not yet from Rhabdomyosarcoma (RMS), the most frequently occurring soft tissue tumor in child-hood. Hence, the aim of this study was to identify and characterize a CSC population in RMS using a functional approach.

We found that RMS cell lines can be passaged in stem cell medium containing defined growth factors over several passages and are capable of forming rhabdospheres. Using an orthotopic xenograft model, we demonstrate that a 100 fold lower number of sphere cells result in tumor growth compared to the bulk population suggesting that CSCs were enriched in the sphere population. Furthermore, stem cell genes such as Oct4, Nanog, c-Myc and Sox2 are significantly upregulated in rhabdospheres which can be differentiated into multiple lineages such as adipocytes, myocytes and neuronal cells upon treatment with appropriate stimuli.

Surprisingly, gene expression profiles indicate that rhabdospheres show more similarities with neuronal than with hematopoietic or mesenchymal stem cells. Analysis of these profiles identified the known CSC marker CD133 as one of the genes also upregulated in rhab-dospheres, both on RNA and protein levels. $CD133^+$ sorted cells were subsequently shown to be more tumorigenic and more resistant to commonly used chemotherapeutics. Using a tissue microarray (TMA) and correlating CD133 expression with clinical data, we found that high expression of CD133 correlates with poor overall survival suggesting that first CD133 could be a prognostic marker for eRMS patients and second such a cell population is also present in human primary tumors.

These experiments indicate that a $CD133^+$ CSC population can be enriched from RMS which might help to develop novel targeted therapies against this pediatric tumor.

2. Zusammenfassung

Krebsstammzellen (KSZ) sind in einigen soliden Tumoren identifiziert worden, bisher jedoch noch nie im Rhabdomyosarkom (RMS), welches das häufigste vorkommende Weichteilsarkom im Kindesalter ist. Aus diesem Grund war das Ziel dieser Studie, eine KSZ - Population mit Hilfe eines funktionalen Ansatzes zu identifizieren und zu charakterisieren.

Wir konnten aufzeigen, dass RMS Zelllinien über mehrere Passagen im Stammzellmedium, das bestimmte Wachstumsfaktoren enthält, kultiviert werden und Rhabdospheren mit steigender Anzahl gebildet werden konnten. Mit einem orthotopischen Xenograft-Modell, konnten wir nachweisen, dass 100fach weniger Spherezellen, die mit KSZ angereichert waren, als Bulkzellen benötigt wurden, um ein Tumorwachstum zu induzieren. Darüber hinaus waren Stammzellgene wie Oct4, Nanog, c-Myc und Sox2 in Rhabdospheren signifikant hochreguliert und ließen sich in verschiedene Linien, wie Adipozyten, Myozyten und neuronale Zellen, nach einem bestimmten Stimulus differenzieren. Interessanterweise, deutete das Genexpressionsprofil-Experiment darauf hin, dass Rhabdospheren mehr mit neuronalen Stammzellen als mit hämatopoietischen und mesenchymalen vergleichbar sind.

Die Analyse der Profile identifizierte einen schon bekannten KSZ - Marker, CD133, der in den Rhabdospheren auf RNA - und Proteinebene hochreguliert war. Ein weiterer Punkt war, dass $CD133^+$ sortierte Zellen mehr tumorigen und resistent gegen Chemotherapie waren.

Um schliesslich die CD133 – Expression mit klinischen Daten vergleichen zu können, färbten wir einen humanen RMS – Gewebe – Microarray und untersuchten die CD133 Verteilung bei verschiedenen Patienten. Hohe CD133 Expression ist mit einer schlechten Überlebenschance korrelierbar, was darauf hinweist, dass erstens CD133 ein prognostischer Marker für embryonale RMS - Patienten sein könnte und zweitens dass unsere *in vitro* gefundene Subpopulation auch in primären Tumoren existiert.

Diese Experimente zeigen auf, dass eine $CD133^+$ KSZ – Population im RMS angereichert werden kann, die zur Entwicklung von zukünftigen getargeten RMS – Therapien beisteuern kann.

3. List of Abbreviations

APC	adenomatous polyposis coli
AKT	V-AKT murine thymoma viral oncogene homolog 1
ALDH1	aldehyde dehydrogenase 1
aRMS	alveolar Rhabdomyosarcoma
bFGF	basic fibroblast growth factor
BCC	basal cell carcinoma
BSA	bovine serum albumin
CML	chronic myeloid leukemia
CIC	cancer initiating cell
CBP	CREB-binding protein
CREB	cAMP response element binding protein
CSC	cancer stem cell
DAPI	4',6-diamidino-2-phanylindole
DHh	desert hedgehog
DMEM	Dulbecco's minimum essential medium
DMSO	dimethylsulfoxid
E-Cad	E-Cadherin
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGTA	ethylene glycol tetraacetic acid
EMT	epithelial to Mesenchymal transition
ERα	estrogen receptor α

ERK	extracellular signal-regulated kinase
eRMS	embryonal Rhabdomyosarcoma
FCS	fetal calf serum
FGFR	fibroblast growth factor receptor
FKHR	forkhead-related
FSH	follicle-stimulating hormone
GPCR	G-protein coupled receptor
GSK-3	glycogen synthase kinase 3
Hh	hedgehog
IHh	indian hedgehog
IGF	insulin-like growth factor
IGFR1	insulin-like growth factor receptor I
IL4	interleukin-4
i.m.	intra muscularly
iPS	induced pluripotent stem cell
JAK	janus kinase
LEF	lymphoid enhancer binding factor
LGR	leucine-rich G-coupled receptor
LH	luteinizing hormone
LOH	loss of heterozygocity
LOI	loss of imprinting
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MMP	metalloproteinase

mRNA	messenger RNA
MSC	mesenchymal stem cell
NOD	non obese-diabetic
NOD/Scid	NOD.CB17-*Prkdc*^{scid}/J
NSG	NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ
ns	statistically non significant
OS	overall survival
PAX	paired box protein
PI3K	phosphoinositide-3-kinase
PBS	Phosphate buffered saline solution
PFA	paraformaldehyde
PKC	protein kinase C
Ptch1	patched receptor 1
RA	retinoic acid
RMS	rhabdomyosarcoma
qRT-PCR	quantitative real-time polymerase chain reaction
SC	stem cell
SHh	sonic hedgehog
siRNA	short interfering RNA
Smo	smoothened
SP	side population
T-ALL	T cell acute lymphoblastic leukemia
TCF	T-cell transcription factor
TGFß	tumor growth factor ß

TIC	tumor initiating cell
TSH	thyroid-stimulating hormone
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

4. Introduction

4.1. Cancer

After decades of research, the mortality of cancer begins to decline significantly. This is mainly due to an increased understanding of the mechanisms leading to cancer development and to fast and successful advances in cancer treatment approaches such as the introduction of combination treatments and targeted therapies, targeting a tumor specific feature such as the tyrosine kinase abl. Abl is activated in chronic myeloid leukemia (CML) by the bcr-abl translocation and specifically blocked by the kinase inhibitor Gleevec (1; 2)

Cancer is a multistep process during which normal cells become malignant through genetic and epigenetic alterations. Many factors are associated with cancer development such as age, body weight, immune system, carcinogens (e.g. chemicals), viruses (e.g. Papilloma virus) and inherited polymorphisms (3; 4). The vast majority of cancers is sporadic and is caused by somatic genetic mutations. There is, however, cancer arising in families, which have natural variants in their genes (polymorphisms) such as mutations activating oncogenes or inactivating tumor suppressor genes (5). Through novel approaches such as high-throughput single nucleotide polymorphism (SNP) array technology, alterations and variations can be detected leading to earlier diagnosis and thus better chances of survival.

Carcinogenesis is initiated by irreversible genetic damages leading to a stable cell clone which proliferates and has abnormal growth rates. In the progression phase more stable cell clones with additional genetic mutations expand owing to an imbalance in cell growth, death and differentiation. Finally, the transformed cells proliferate extensively and acquire the potential to invade the surrounding tissue and metastasize (Figure 1).

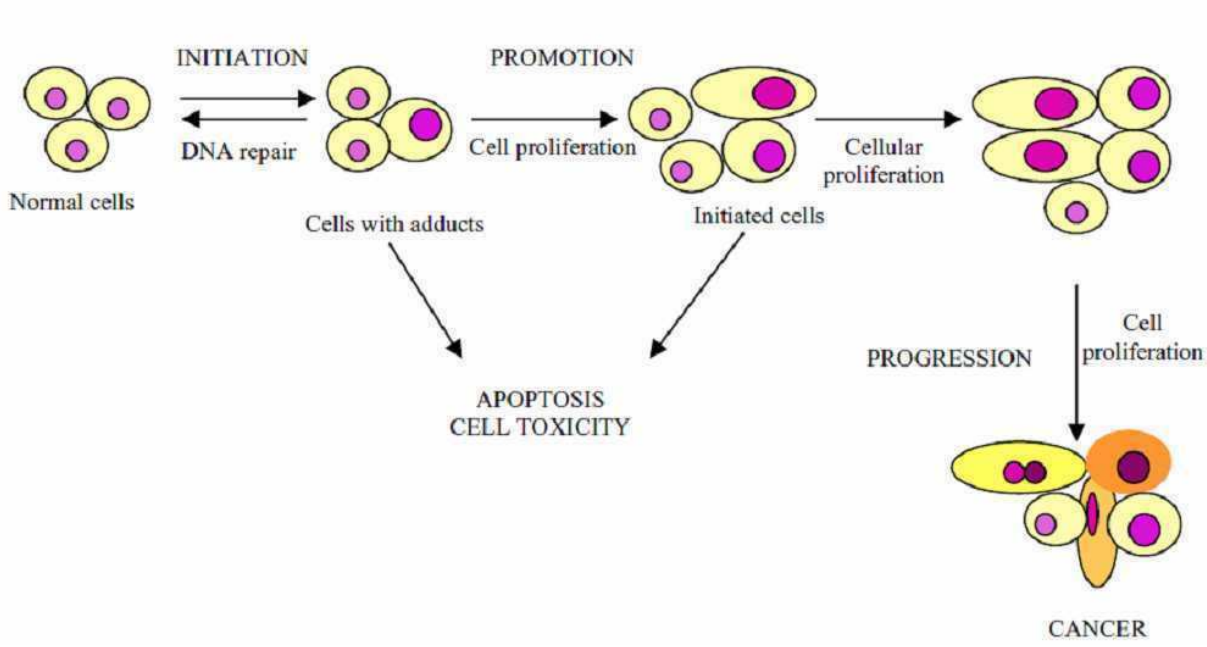

Figure 1: Carcinogenesis is a multistep process: initiation, promotion and progression. Irreversible genetic and epigenetic alterations are leading to expansion of tumorigenic cell populations having uncontrolled growth, invasion and metastasis potential (4).

Cancer is a heterogeneous group of diseases which can affect any part of the body and is characterized by a set of functional capabilities, so-called hallmarks of cancer like self-sufficiency in growth, insensitivity to anti-growth signals, tissue invasion and metastasis, sustained angiogenesis and evasion of apoptosis (6) (Figure 2A). The definition of hallmarks of cancer was further expanded during the last ten years. Kroemer et al. proposed shutting down of the immuno surveillance as the seventh hallmark of cancer (7). Furthermore, the induction of stress factors like mitotic and metabolic stress, leads to genomic instability (8). Finally, Negrini summarized all hallmarks and suggested the first steps causing carcinogenesis (9) (Figure 2B). In sporadic cancer, genetic as well as epigenetic alterations lead to extensive proliferation rates, evasion of apoptosis and development of a stable cell clone. In contrast, polymorphisms and several mutations which can be inherited are responsible for proliferation, angiogenesis, DNA damage and evasion of apoptosis in hereditary cancer.

Nevertheless, the cell of origin and the forces driving tumorigenesis are still unknown for most cancer types. It is important to know how a tumor arises, which cells are tumorigenic and what the role of the microenvironment is. There are two models addressing these unre-

solved questions: the clonal evolution and the cancer stem cell model being discussed in the next chapter.

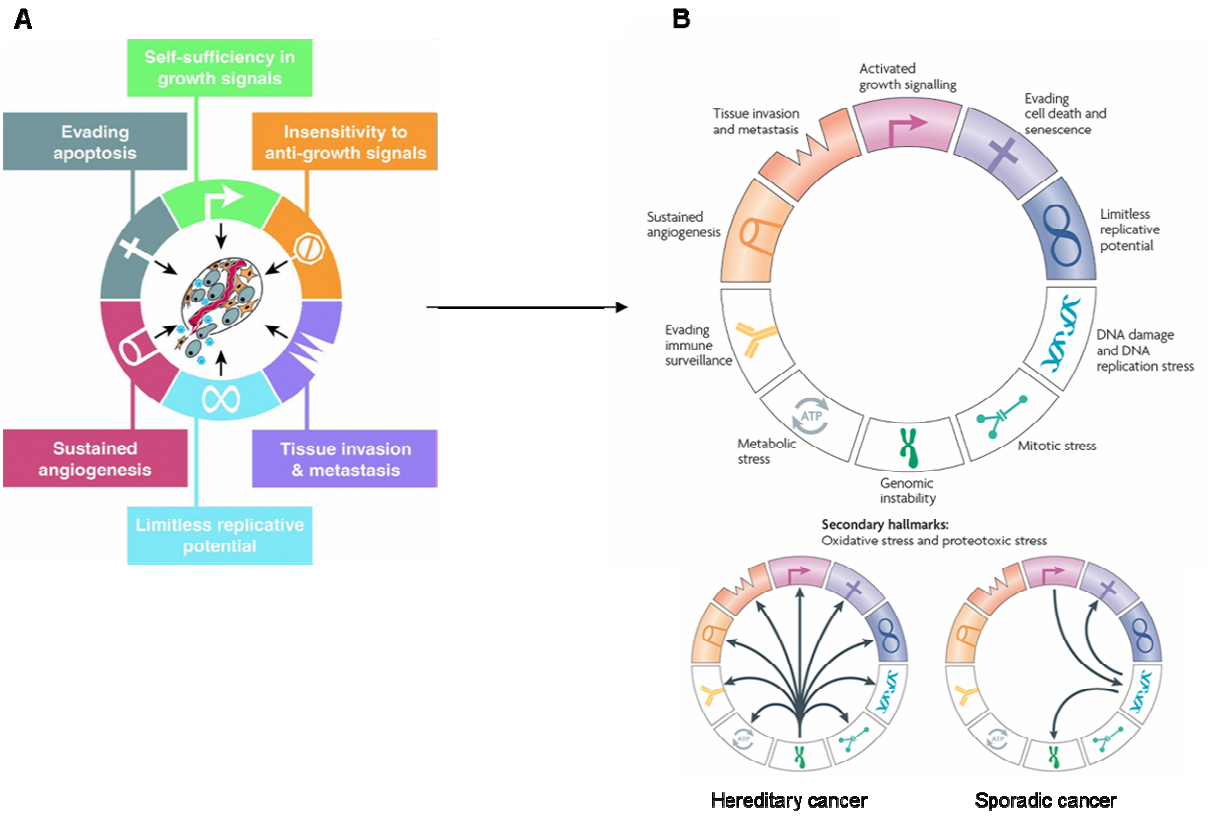

Figure 2: Hallmarks of cancer.
Described in 2000 from Hanahan et. al (10) and revised by Negrini et. al (9).

4.2. "Clonal evolution" versus "Cancer stem cell"

One hallmark of the clonal evolution model, also known as stochastic cancer model (Figure 3A), is that genetic and epigenetic alterations generate stable tumorigenic cell clones responsible for tumor formation. Thus, tumors have a heterogeneous morphology displaying each malignant cell clone and are not necessarily organized hierarchically. Furthermore, the clonal evolution model predicts that each cell within a tumor is potentially a tumor initiating cell (TIC). Hence, the tumorigenic mechanisms are operative in all bulk tumor cells from which the key properties can be identified, and injection of a single tumor cell *in vivo* should lead to tumor growth, since each cell has tumor initiating capabilities. The logical consequence of this model is that the rational approach to treat tumors is to target the bulk of the cancer cells (11; 12).

In contrast to the clonal evolution model, the cancer stem cell (CSC) model predicts that tumors are organized hierarchically and thus have a heterogeneous phenotype (Figure 3B). Only a limited number of cells are capable of tumor formation and these TICs have specific mechanisms which regulate the tumorigenic potential (13). One hallmark of the CSCs or TICs is the self renewal capability which is controlled by signaling pathways shared or present also in normal stem cells (SCs), like Hedgehog, Notch and Wnt, and by the tumor specific niche, which is a specific microenvironment surrounding the tumor that can activate specific signaling pathways through cell-cell interaction (see also “Embryonic signaling pathways”) (14-20). Additionally, this model predicts that CSCs are more resistant to chemotherapy than the bulk tumor cells and are therefore responsible for tumor recurrence (21-26).

According to this model, after isolation of the different tumor cell subpopulations and injection into mice, only the CSC subpopulation should lead to tumor formation (27). Thus CSCs have the potential to proliferate extensively *in vitro* and *in vivo*. One consequence of the CSC model is that treatment approaches should target primarily the resistant CSCs in order to generate more specific and effective therapies (28).

In the past years, the study of CSCs has gained importance in cancer research. Nevertheless, open questions such as how CSCs develop which stem cell properties they possess, how they differ from normal stem cells, and what their exact role in drug response and tumour formation is, need to be addressed to further understand the biology of CSCs.

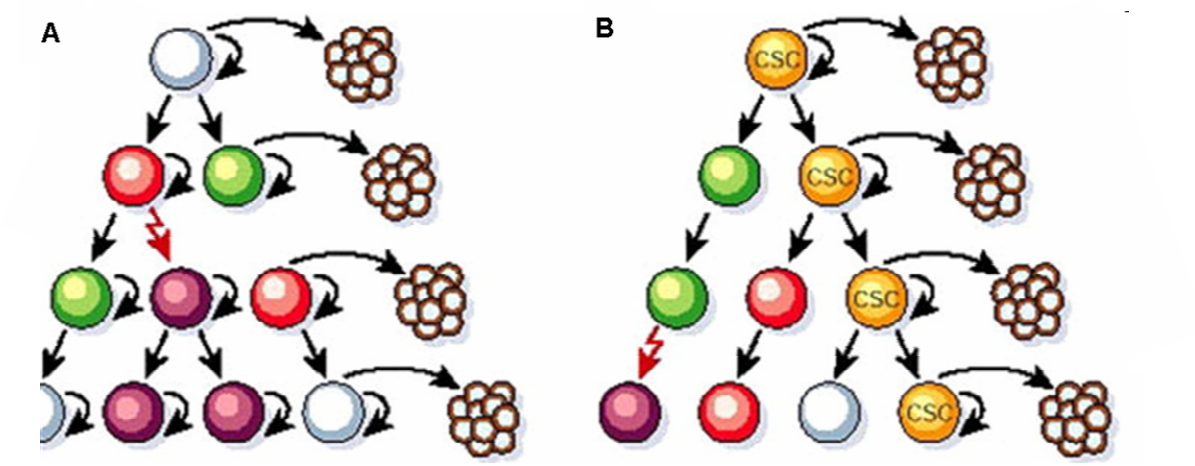

Figure 3: Clonal evolution model versus cancer stem cell model.
A) In the clonal evolution model, tumors are not necessarily organized hierarchical; a lot of cells are tumorigenic and can lead to tumor formation. B) In the CSC model, only a small subpopulation of cells is tumorigenic and responsible for tumor formation and thus controls tumor development (13).

4.3. Cancer stem cells

More than 50 years ago Makino hypothesized that a small subpopulation sharing common characteristics with normal stem cells (SCs) - such as the probability to self renewal, the potential to differentiate, extensive proliferation, the resistance to chemotherapeutics - is responsible for tumor development (27) and that tumors are organized hierarchically. These so-called cancer stem cells (CSCs) were first identified in acute myeloid leukemia (AML). AML cells, being $CD34^{+}/CD38^{-}$, had a limited proliferative capacity indicating that this tumor arises from one stem cell (29; 30). It was therefore not surprising that CSC populations were also identified in solid tumors being hierarchically organized (33-35). At first in breast cancer, a $CD44^{+}/CD24^{-/low}$ CSC population was identified and characterized (36). Subsequently, CSCs were identified in several other tumors such as, among others, brain tumors (37-42), colon cancer (43-45), and melanomas (46).

Despite the identification of CSCs in several tumor types, how CSCs arise remains unclear (Figure 4). One hypothesis is that CSCs derive from normal stem cells (SC) or progenitor cells through genetic mutations to which they are more prone because of their long life span. Another hypothesis is that differentiated cells can de-differentiate through several genetic and epigenetic alterations and thus acquire a stem-cell like phenotype with some stem cell properties (47). Therefore, the CSC can be a normal stem cell, a progenitor cell or an already differentiated cell. The implication of these hypotheses is that there is not one CSC common

to all tumors, and that it will be necessary to first identify the CSC population for any given tumor and then to characterize it in more detail to be able to design specific therapies.

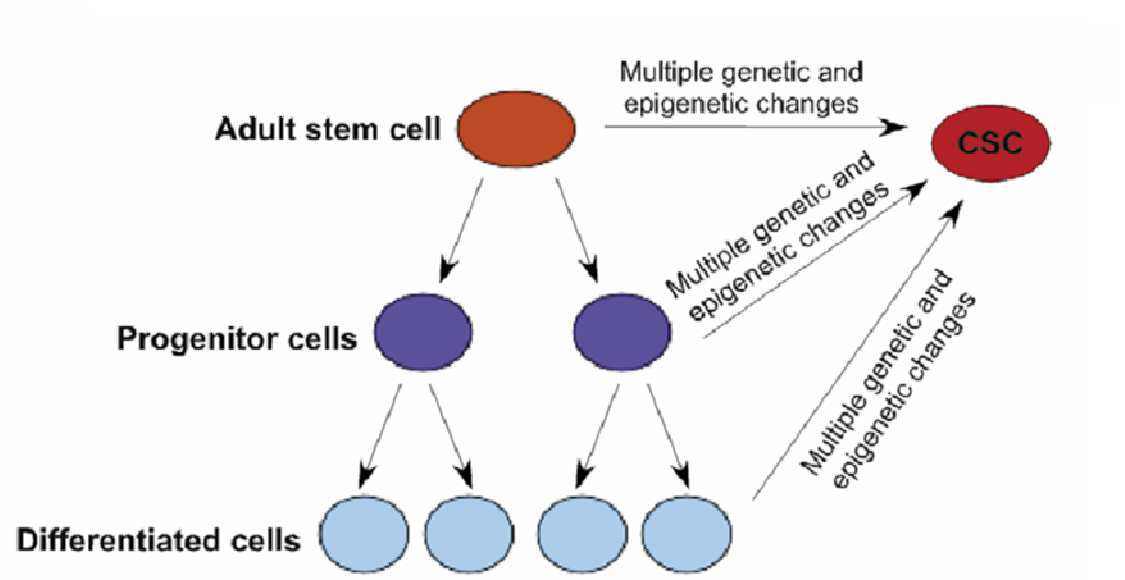

Figure 4: Development of CSC.
CSC can arise from adult stem cells and progenitor cells through several genetic and epigenetic alterations. Moreover, differentiated cells can dedifferentiate to a cell population with a stem-cell like phenotype (47).

The “gold standard” to define a CSC population is to divide tumor cells into subpopulations and to perform limiting dilution assays *in vivo* by injecting each subpopulation into mice and subsequent monitoring of tumor growth. Furthermore, enrichment of a subpopulation with self renewal property *in vitro* by sphere assays originally developed for isolating neuronal SC is also accepted as indicator for the presence of CSCs (48). Several known SC markers are now recognized as CSC markers in cancer which will be further described in the text (see “Known cancer stem cell markers”) (27). After injection of the different subpopulations, tumor growth should take place only in mice injected with the CSC subpopulation and in bulk injected mice (control mice). The most commonly used mice are immunocompromised mice, like SCID and nude mice for example. In addition, yet other mouse models that were developed for the study of tumor biology and mechanisms leading to cancer seemed useful for studying CSCs (49). One example is the Patched-1-deficient mouse model of medulloblastoma, where Ward et al. detected a highly proliferating and tumorigenic CD15 positive subpopulation in this mouse model which was responsible for tumor formation (50).

To summarize, in the last decades, CSCs were detected in various tumor types and several mouse models are available to study development of these tumors.

However, controversial results exist in different tumor types such as melanoma (51-55), brain tumors (38; 56-58) and colon cancer (13; 16; 21). For example, CD133 is a marker describing the subpopulation of CSC in brain tumors, but in tissue sections from human tumors, only a subpopulation of patients had a $CD133^+$ CSC population (38; 56-58). Furthermore, various markers describing different subpopulations were identified in colon cancer, and it is not yet clear which ones or which combination thereof describes the CSC population (16; 21; 59).

Hence, following questions remain: Are tumors hierarchically organized or not? Which markers are describing the CSC population in a given tumor and are there universal CSC markers? Are there more tumor initiating cell populations in one tumor? Which mouse model should be chosen (12; 52)?

Recently, a new model combining the clonal evolution and the CSC model has been proposed (see Figure 5) to answer some of the remaining questions. According to this model, once the normal SC pool is activated and epigenetic as well as genetic alterations are acquired, the SCs develop into CSCs. These CSCs share common characteristics with the normal SCs they originate from and are responsible for tumor growth. Additional mutations lead first to a stable clone of CSCs with self renewal properties, and then the interaction with the “tumor niche” drives the expansion of several mutagenic clones, each one being a tumor initiating cell (TICs). Therefore, in this model the frequency of TICs is relatively high and the model explains why metastasized tumors have a different CSC population then their primary tumors. Accordingly, treatment approaches should target both, primary tumor and metastasis, or by blocking the invasion process.

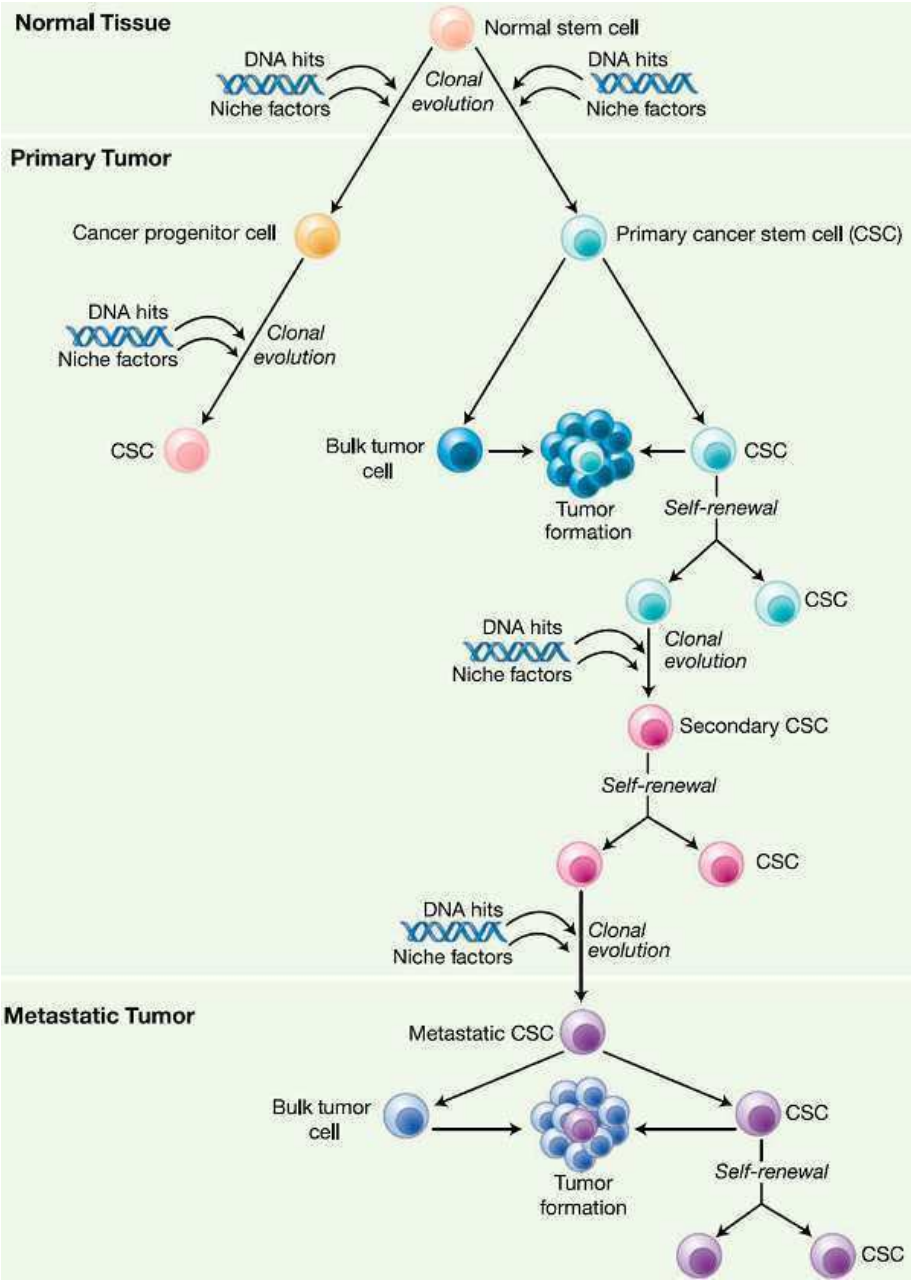

Figure 5: A new cancer model: Combination of the clonal evolution and CSC model
Epigenetic and genetic alterations are activating the SC pool and thus leading to uncontrolled cell growth which is hierarchically organized. Further mutations are responsible for the expansion of a stable cell clone (TIC) (60)

A further point which should be discussed is the resistance to commonly used therapeutics and how the CSC model can help to explain this resistance. It has been shown that CSCs are more resistant to chemotherapy and radiation and by careful characterization of these subpopulations, new treatment approaches can be established (21; 22; 28; 37; 44; 61; 62). Todaro et al noticed that the CSC of colon cancer is $CD133^{+}$ and expresses and secretes high levels of interleukin-4 (IL4). The use of a neutralizing antibody against the IL4 receptor, blocking the binding site of IL4, leads to less chemoresistant CSCs (44; 45; 63). Further-

more, the niche embedding the CSCs can play a role in drug resistance (64; 65). Therefore, disrupting the interactions between niche and CSC can be crucial for overcoming therapeutic resistance (66-69). Looking into the pathways specifically upregulated in the CSC population, some groups could show that by inhibiting the PI3K/AKT pathway, the CSC got more sensitive to different treatment approaches (61; 70; 71).

In summary, these findings allow the following conclusion: before treating a tumor, it should be defined which model the tumor follows. Answering this question will result in better and gentler treatment approaches.

4.3.1. Known cancer stem cell markers and assays

In the late nineties, Lapidot and Bonnet identified a leukemia initiating subpopulation in AML which was positive for CD34 and negative for CD38. They could show that only the $CD34^+/CD38^-$ cells can initiate tumor growth *in vivo*. Thus, they concluded that the origin of the CSC in leukemia is a $CD34^+$ hematopoietic progenitor cell (31; 32). The search for the origins of cancer stem cells in solid tumors led to the discovery of several marker proteins and assays being based on high expression levels of multidrug resistance receptor cells, some of have a role in development and are now known as cancer stem cell markers describing a subpopulation with self renewal properties. Here, I will briefly introduce the established and accepted CSC markers and assays.

4.3.1.1. CD24

CD24 is a glycosyl phosphatidylinositol (GPI) linked cell surface protein and is expressed on B lymphocytes and differentiating neuroblasts. Furthermore, it's highly glycosylated mostly with O- and N-glycosylations. Lack of CD24 is associated with E-cadherin repression and Snail, Twist and TGFß3 upregulation which induces a mesenchymal phenotype in epithelial cells, whereas overexpression of CD24 and thus downregulation of Snail, Twist and TGFß3 is associated with an epithelial phenotype. Furthermore, CD24 negative cells are associated with highly invasive cells and CD24 positive cells play an important role in cell adhesion and cell-cell contacts (72).

In breast cancer, a more tumorigenic subpopulation positive for CD44 and negative or low positive for CD24 ($CD44^+/CD24^{-/low}$) was described and this subpopulation had a more mes-

enchymal phenotype (73). Moreover, expression of CD24 in breast cancer is correlated with the expression of the estrogen receptor α (ERα). Patients expressing high levels of ERα have low or no CD24 expression underscoring the concept that CD24 low or negative cells have a mesenchymal phenotype (74). However, pancreatic tumors have an epithelial phenotype implying a non- or low metastatic potential of the CSC population, but the identified CSC population is CD24 positive ($CD24^+/CD44^+/ESA^+$) and highly metastatic (75-77). Therefore, CD24 expression can be associated with an epithelial or mesenchymal phenotype and its role in carcinogenesis is tissue specific.

4.3.1.2. CD44

CD44 isoforms are single-pass type I transmembrane proteins consisting of a large extracellular domain, a stem structure, a transmembrane domain and a cytoplasm tail (Figure 6). These proteins are encoded by the same gene and through alternative splicing, several different isoforms are generated. The smallest protein, also known as the standard CD44 (sCD44), has no variant exons and is expressed in the whole body. In contrast, through splicing of the variant exons, larger CD44 proteins are produced and are mainly expressed in epithelial or tumor cells. CD44 can interact with hyaluronic acid (hyaluronan), osteopanin, collagens and matrix metalloproteinases (MMPs) among others. Its function is modified by posttranslational modifications like glycosylation and can be separated into lymphocyte activation, recirculation, homing and hematopoiesis. Uncontrolled transcription of CD44 can lead to aberrant expression and thus its overexpression is correlated with enhanced tumor growth, survival, motility and invasiveness (78-82). Moreover, CD44 expression is associated with some CSCs in breast (83; 84), prostate (85), colon cancer (86) and leukemia (87). However, CD44 expression in cancer is not always correlated with poor prognosis. Notably, in prostate cancer low CD44 expression or absence of CD44 correlate with poor prognosis. There, overexpression of CD44 leads to a reduced metastatic potential (88).

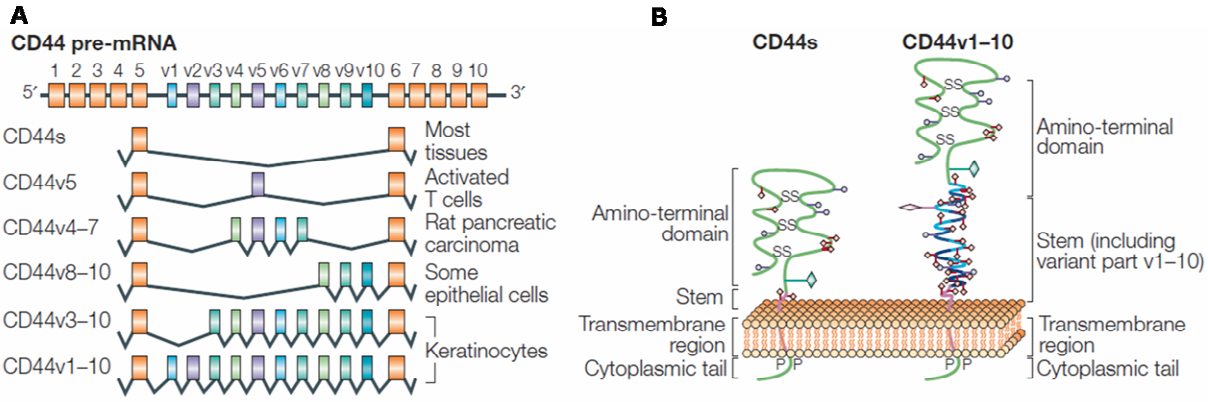

Figure 6: CD44 proteins
A) Splicing variants of CD44. B) Structure of standard CD44 (sCD44) and larger CD44 (78)

4.3.1.3. CD133

CD133, also known as Prominin1, is a five transmembrane protein with eight potential N-glycosylation sites. It was first described in murine neuroepithelial cells and in human it was detected as a hematopoietic SC marker. Hematopoietic $CD34^+$ progenitor cells are also positive for CD133 (89). The function of CD133 is still unknown, but it was shown that CD133 is also a CSC marker in brain tumors (38; 40; 58; 90; 91), breast (92), colon (44; 45; 61; 93; 94), pancreatic (95; 96), liver (62; 97), skin (51; 53) and prostate cancers (98; 99) and Ewing's sarcoma (100). Furthermore, $CD133^+$ glioma stem cells are more resistant to chemotherapy and radiation than bulk and the CD133 negative population (22; 24; 25). CD133 downregulation induced differentiation in neuroblastoma cell lines and hence increased sensitivity to drug treatment (91). Nevertheless, it is not clear, whether a $CD133^+$ subpopulation is describing the CSC population in melanoma or prostate cancer (12; 52; 101).

4.3.1.4. LGR5

G-protein coupled receptors (GPCRs) are a large family of seven transmembrane receptors consisting of leucine-rich repeat domains on the extracellular site and a transmembrane and an intracellular domain. The intracellular part is responsible for signaling (Figure 7). More than a decade ago, Hsu described two proteins sharing common features with the hormone receptor for thyroid-stimulating hormone (TSH), follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These receptors were named leucine-rich repeat G-protein coupled receptor 4 and 5 (LGR4, LGR5) (102). Two years later, a new member, LGR6, was cloned (103). One member of the leucine-rich repeats GPCRs, LGR5, was recently described as a

marker for intestinal SC. Crypt cells of the intestine were positive for LGR5 and these $LGR5^+$ cells differentiate into intestinal cells and mucosa (104). Subsequently, Jaks et al. detected $LGR5^+$ cells in the hair follicles and could show that these cells had a high proliferation rate after activation (105). Furthermore, $LGR5^+$ cells showed upregulation of several Wnt-target genes. By knocking down or inactivating APC as a target of Wnt signaling positive cells were demonstrated to be responsible for tumor development. Thus LGR5 is an established CSC marker in several tumors with mutations in the Wnt pathway (106-111).

It was recently described in basal cell carcinoma (BCC) that LGR5 is a target of the hedgehog pathway (112). BCCs have mutations in the Patched receptor leading to a constitutively active pathway and thus to increased proliferation rates (113). In the late nineties Hahn et al. developed a mouse model with a heterogeneous Patched mutation to investigate whether active hedgehog could trigger tumor development. These mice developed BCC, RMS and medulloblastoma (114-116); anyhow, only 10% of the mice developed RMS. Therefore, for studying the hedgehog pathway and its target LGR5 in RMS a mouse model with higher incidences is needed. Mao et al. developed a mouse model, where smoothened, a downstream target of hedgehog, is mutated. After induction of tumor growth with tamoxifen, the incidence of RMS was dramatically increased (117).

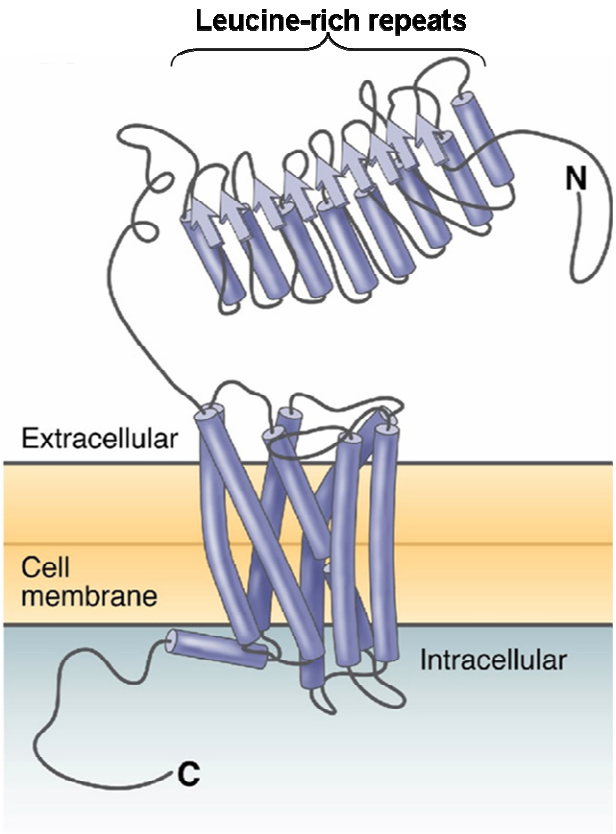

Figure 7: Predicted structure of leucine-rich repeat G-protein coupled receptors
Leucine-rich repeats mark the extracellular domain. The intracellular and the transmembrane parts are responsible for signaling (102).

4.3.1.5. Hoechst 33342 dye efflux

Even though known CSC markers are still controversially discussed, it might be possible to identify a general marker describing SCs and CSCs in several tumors. Goodell et al. detected a subpopulation of hematopoietic cells which were less positive after Hoechst 33342 staining than the rest of the population. Hoechst 33342 intercalates into DNA of viable cells and can be detected by flow cytometry (118). Further analysis revealed that this subpopulation is highly positive for CD34 which is a marker for hematopoietic progenitor cells. Transplantation of this subpopulation into lethally irradiated mice leads to reconstitution of the hematopoietic system. Moreover, there was no subpopulation detectable after blocking the ABC transporter MDR1 with Verapamil. Finally, they named this subpopulation a side population (SP) with self renewal property and differentiation potential (119).

Hence, SPs were described in neuronal SC and progenitor cells (48) and finally, SP with SC property or a stem-cell like phenotype were detected in leukemia (120) and several other tumors, for example brain and prostate tumors (121-130). However, one disadvantage of the Hoechst 3342 dye is that it is mutagenic since it can disrupt the DNA replication and cell division.

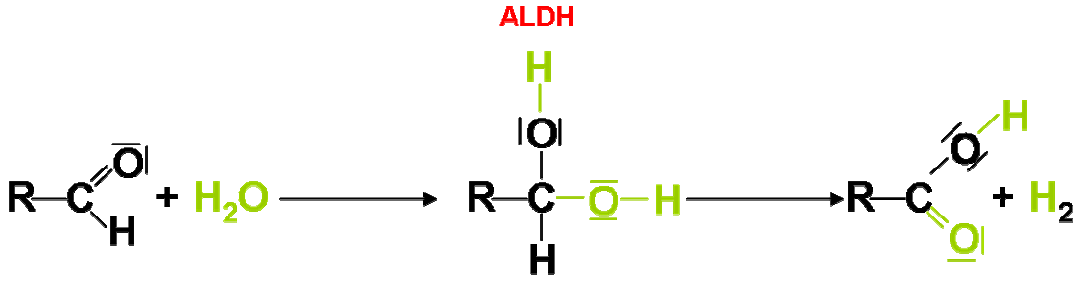

Figure 8: Oxidation of an aldehyde catalyzed by an aldehyde dehydrogenase

4.3.1.6. ALDH1 activity

It is known that many cells express the enzyme aldehyde dehydrogenase (ALDH) which catalyzes the oxidation of an aldehyde into a carboxy acid (Figure 8). To date, 17 isoforms of this enzyme have been identified. Most of them are widely expressed in the body and have various substrates in different tissues. For example, ALDH1 catalyzes the reaction of retinal (Vitamin A) into retinoid acid (RA) in the liver (131; 132). Furthermore, $CD34^+$ hematopoietic progenitor and intestinal crypt cells are highly positive for ALDH1. Thus, designing an assay depending on intracellular ALDH1 activity for sorting these cells was indispensable was highly desirable. Jones et al. described a fluorescent substrate for ALDH1 which can be detected by fluorescence activated cell sorting (FACS). However, the emission wave length was in the ultraviolet range and thus by itself mutagenic for cells (133). To avoid this effect, Storms et al. developed a new system with a fluorochrome in the visible light range which they named Aldefluor (134). Subsequently, CSCs positive for ALDH1 were characterized in leukemia, breast, prostate, pancreas cancer and head and neck squamous carcinoma (135-139).

4.3.2. Embryonic signaling pathways

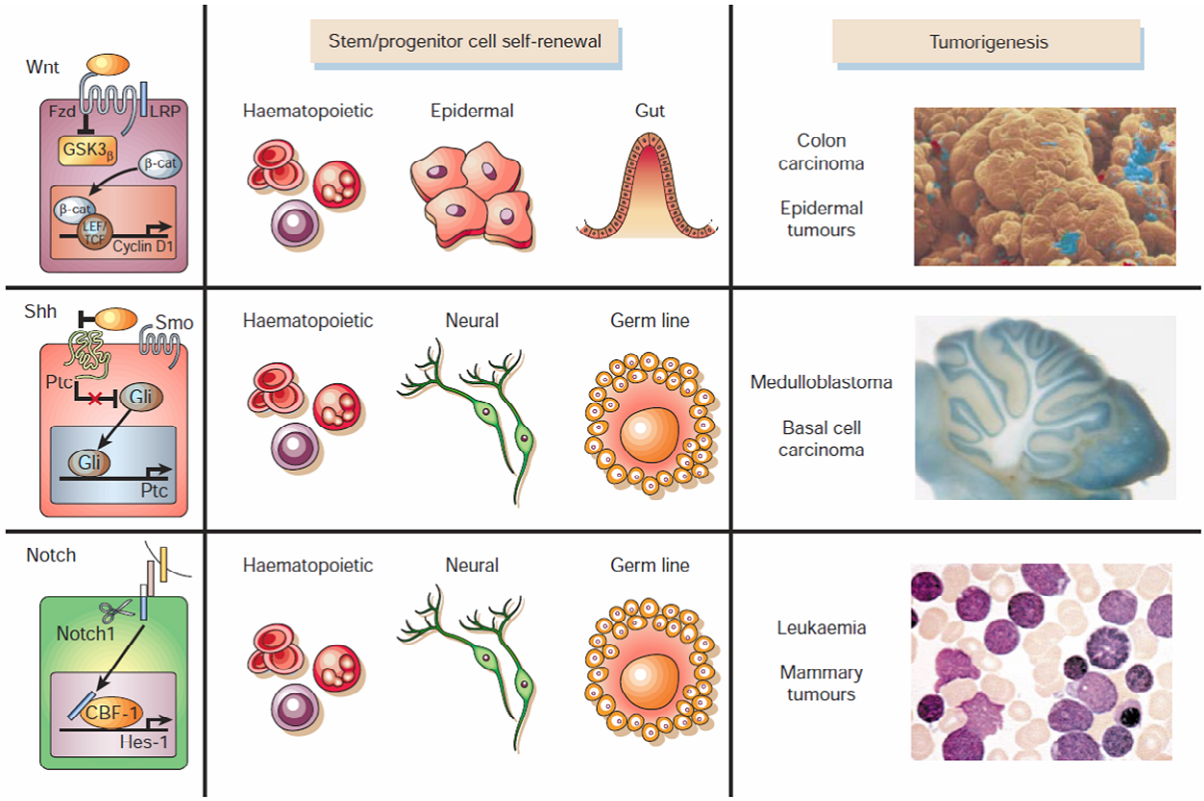

Figure 9: Embryonic signaling pathways regulate the activation of stem and progenitor cells with self renewal capability and can drive tumorigenesis.
Notch and SHh signaling pathways are involved in hematopoiesis; neurogenesis and germ line development, whereas Wnt activates hematopoiesis and epidermal and gut development. Continuous activation of these pathways may lead to uncontrolled growth rates and is linked to tumorigenesis (140).

During embryonic development, different signaling cascades (Figure 9) have to be activated in SCs, for then to proliferate and divide asymmetrically into one progenitor cell with differentiating potential and a SC. One important step in the activation of SCs is crosstalk between the niche and SC, thus embryonic signaling pathways like Hedgehog (Hh), Notch and Wnt are activated by their respective ligands and lead to higher but controlled proliferation rates. On the contrary, tumor cells have abnormal growth rates and proliferate extensively and it has been shown that in some cancers embryonic signaling cascades are activated by the crosstalk with stroma (tumor niche) leading to cell proliferation, resistance, invasion and metastasis. Therefore, blocking this interaction should circumvent resistance (60)

4.3.2.1 Hedgehog signaling pathway

The Hedgehog pathway was initially identified in drosophila development. It is active during segmental pattering. In mammalians, it is highly active and necessary specifically for neural tube and skeleton development and silenced in most adult tissues, where the pathway is only activated during tissue repair. Normally, the 12 transmembrane (TM) receptor Patched (PTCH1) represses constitutively active Smoothened (SMO), a seven TM receptor with homology to GPCRs. If ligands such as Sonic (SHH), Desert (DHH) or Indian (IHH) hedgehog bind to PTCH1, SMO is released and activates GLI1, 2 and 3, which are transcriptional regulators. The balance of the Gli activation is important for expression of target genes. Furthermore, Gli1 can activate Snail1 which represses expression of E-cadherin (E-Cad) in epithelial cells and thus favors a more mesenchymal phenotype. This mechanism is named epithelial to mesenchymal transition (EMT) and is connected to invasion and metastasis in cancer. For example, colon tumors which express high levels of Gli1 are highly metastatic. Inhibition of Snail1 activation by cyclopamine or siRNA treatment leads to Snail1 reduction and thus a reduced tumor growth (141).

Therefore, the question remains how the Hh pathway is activated in cancer. Different mutations can lead to constitutively active Glis for example (142; 143) (Figure 10A). In addition, tumor cells produce the Hh ligands by themselves and thus activate the pathway in an autocrine manner (Figure 10B) which has been described in several tumors (see also Table 1). As mentioned above, crosstalk between stroma and tumor cells is also important in activation of the Hh pathway. Therefore, stroma cells can produce the Hh ligands which activate this pathway in tumor cells (paracrine activation; stroma to tumor) (Figure 10C) or tumor cells produce and secrete the Hh ligands activating the Hh pathway in stroma cells leading to the induction of target genes such as IGF2 and VEGF which induce proliferation or invasion of tumor cells (Figure 10D).

Recent data suggest that the Hh pathway regulates CSCs (144). Disruption of the Hh signaling by SMO mutation in a chronic myeloid leukemia (CML) mouse model resulted in repression of the fusion protein BCR-ABL resulting in inhibition of BCR-ABL$^+$ leukemia SC and a prolonged survival of the xenografts (145). Furthermore, human mammary SC proliferation can be blocked by inhibiting the Hh pathway by cyclopamine or specific siRNA targeting GLI. Blocking the pathway in these cells leads to reduced levels of BMI1, which is a known regulator of normal and tumorigenic mammary SCs (146).

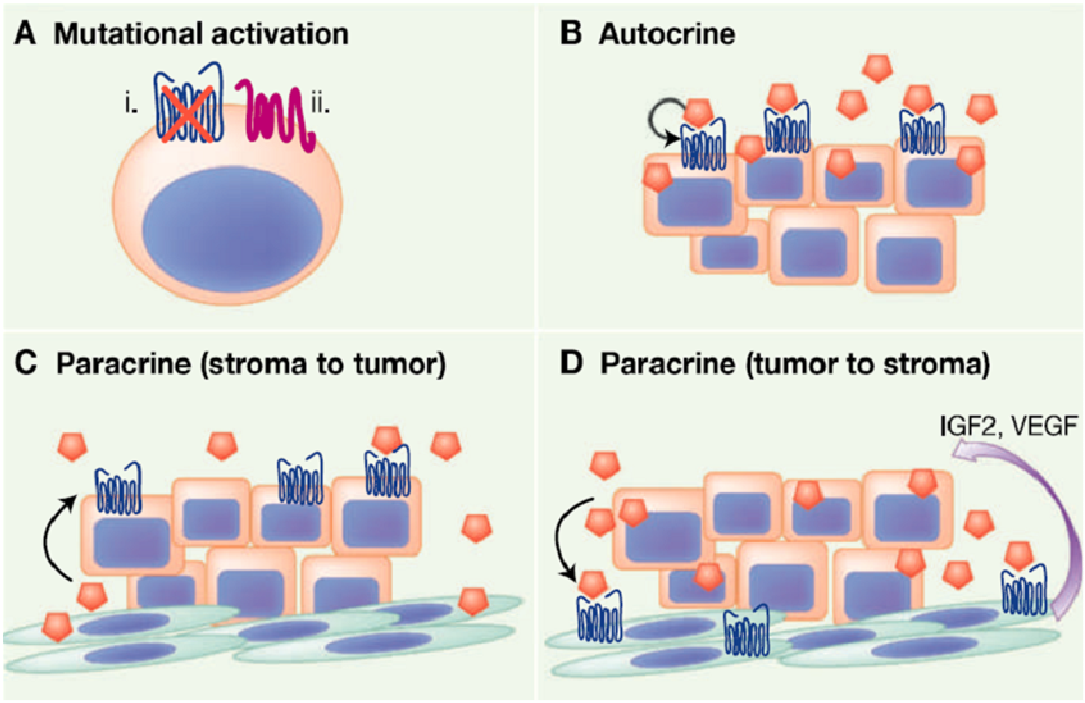

Figure 10: Models of Hedgehog signaling and activation in cancer.
A) The pathway can be directly activated by mutations. B) The most common feature is the production of the Hh ligands by the tumor cells leading to an autocrine activation of the Hh pathway. C,D) Paracrine activation is dependent on the interplay between stroma and tumor. Stroma cells secrete the Hh ligands which activate the pathway in tumor cells (C; stroma to tumor) or tumor cells secrete the ligands activating the pathway in stroma cells which produce target genes that are important for proliferation, invasion, and metastasis (D; tumor to stroma) (144).

In summary, the Hh pathway is an important regulator of embryonic development and is active in adult tissue regeneration. In tumors, the pathway is constitutively activated leading to proliferation of CSCs, invasion and metastasis. Inhibiting Hh pathway activation might be a promising approach for cancer treatment.

4.3.2.2 Notch signaling pathway

Activation of the Notch pathway is important during hematopoiesis, in embryonic development and in adult tissue repair. The Notch pathway can be activated by direct cell-to-cell contact between the membrane bound ligands and the receptors. Five ligands are known, Delta1, 3 and 4 and Jagged1 and 2 and four receptors, Notch1 – 4. Binding of the ligand drives the proteolytic cleavage of the intracellular tail of Notch (ICN/NICD) which translocates to the nucleus. There, it interacts with other DNA-binding proteins and thus Notch specific target genes like HES1 are activated, resulting in enhanced proliferation and block of differentiation and apoptosis.

In cancer, Notch receptors are frequently mutated leading to inappropriate Notch signaling activation. It was shown that in T-cell acute lymphoblastic leukemia (T-ALL), embryonic brain tumors and gliomas, Notch plays a crucial role in the CSC population (20). For example, inhibiting Notch pathways in medulloblastoma SC increased the differentiation potential as well as the sensitivity to chemotherapeutics and xenografts showed a reduced tumor growth (69). Furthermore, $CD133^+$ glioma SCs are more sensitive to irradiation and less tumorigenic after blocking the Notch pathway (67; 68). Thus, disrupting or blocking the interaction between ligand and receptor is under intense investigation and can be used for cancer therapy (Table 2). However, Notch receptors can also have opposing effects as they can act as tumor suppressors. Hence, it is indispensable to study the mechanism of Notch activation in each tumor before blocking it (147-149).

Table 1: Cancers associated with Hedgehog signaling
Abbreviations: NR, not reported; MM, mouse model; P, preclinical; CT, clinical trial (144)

Tumor Type	Mutation-Ligand Mediated (paracrine-autocrine)	Stroma involved in signalling?	Required for CSC?	Type of data
Multiple myeloma	ligand-autocrine paracrine (stroma tumor)	Stroma to tumor	yes	P
Meulloblastoma	mutation lilgand	NR	NR	MM P CT
Upper GI	ligand	NR	NR	P
Pancreatic	ligand-autocrine epithelial cell intrinsic paracrine (to stroma)	Data support Intrinsic requirement and paracrine signalling to signalling to stroma	yes	MM P
Ewing's Sarcoma	non Smo, direct activation Gli1	NR	NR	P
Melanoma	nonligand, nonmutation	NR	NR	MM P
Glioma	ligand-paracrine (exogenous to tumor)	possibly	Yes	P
Breast	ligand, both autocrine and paracrine	possibly	yes	MM P
Ovarian	ligand	NR	NR	P
Prostate	ligand-autocrine	NR	yes	MM P
Small cell lung	ligand	NR	no	MM P
Lymphoma	ligand-autocrine and paracrine	Stroma signals to tumor	NR	P
Leukemia / CML	upregulation of Smo, no mutation ligand-NR	no	yes	MM P
BCC	mutations in Ptch and Smo ligand driven mouse model	no	NR	MM P CT

Table 2: Notch-targeting agents
adapted from (20)

Agent	Mechanism	Targets	Development phase
GSIs:MK0752 (Merck) R04929097 (Roche) PF-03084014 (Pfizer) LY450139 (Eli Lilly) BMS-unknown (BMS)	Inhibition of final Notch cleavage by y-secretase	All 4 Notch paralogs; Notch ligands; Multiple other y-secretase substrates	Phase 1-2
GSMs: MPC-7869 (Myriad)	Inhibition of final substrate cleavage by y-secretase	Selective for specific y-secretase substrates	Unseuccessful phase 3 for MPC-7869, discovery for Notch-targeted GSMs
MAML1-stapled peptid	Interference with Notch nuclear coactivator MAML1	All 4 Notch paralogs, potentially other nuclear transcription factors that use MAML1	preclinical
Notch mAbs	Interference with ligand-induced Notch subunit separation	Specific for individual Notch receptor	preclinical
DLL4 mAbs	Inferference with ligand-receptor interaction	Specific for delta-4 ligand	preclinical
Other ligand mAbs	Interference with ligand-receptor interaction	specific for other Notch ligands	preclinical
Notch soluble receptor decoys	Interference with ligand-receptor interaction	Relatively specific for Notch paralogs potential pan-Notch inhibition	preclinical
siRNA, miRNA-base therapeutics	Interference with expression of Notch signalling components	Specific for target mRNAs	preclinical

4.3.2.3 Wnt signaling pathway

The Wnt pathway is among the most studied, because it was first described in Drosophila as an important pathway for wing development (150; 151). It controls cellular processes like proliferation, differentiation, motility, survival and / or apoptosis, embryonic development and tissue homeostasis contributing to the establishment of cellular polarity. Two pathways are known, the canonical and the more recently described non-canonical Wnt pathway. Both pathways are activated by ligand binding to one of the Frizzled receptors leading to Dvl activation. In the canonical pathway, ß-catenin expression is maintained at low levels and regulated via a multiprotein complex containing Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3ß (GSK-3ß). Ligand binding to Frizzled / low density lipoprotein (LRP) receptor activates Dvl and therefore inhibits GSK-3ß resulting in active ß-catenin. Non-phosphorylated ß-catenin is translocated to the nucleus and forms a complex with the T-cell transcription factor (TCF) and lymphoid enhancer binding factor (LEF). For transcriptional activation, the complex also needs the cAMP response element binding protein (CREB)-

binding protein (CBP) or its homolog p300, to activate the expression of target genes (Figure 11) (152; 153).

Contrary to the canonical pathway, in the non-canonical pathway active Dvl interacts with Ca^{2+} and protein kinase C (PKC) or via Rho/Rac and Janus kinase (JNK) (154; 155). The Wnt pathway is complex and diverse because there are 19 Wnt members and 10 members of Frizzled receptors known and hence different combinations of ligand and receptor lead to various downstream effects (18).

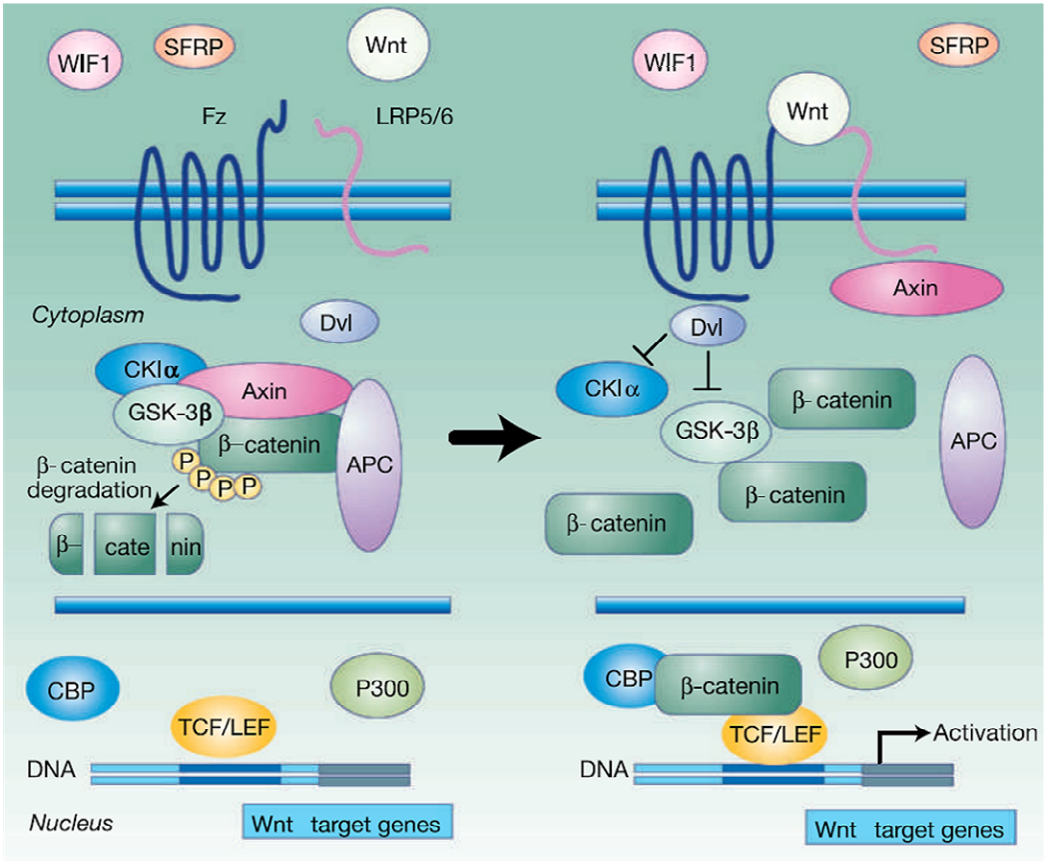

Figure 11: Canonical Wnt signaling pathway
After activation of the Wnt pathway, ß-catenin is translocated to the nucleus, builds a complex with CBP and TCF/LEF to activate the transcription of target genes (18).

Mutations in the Wnt pathway, like mutated ß-catenin being permanently active and overexpressed in several tumors, are driving transcription of cell proliferation genes and thus lead to abnormal growth rates (18; 156-158). Recently, the Wnt target LGR5 was described as a CSC marker in intestinal and colon cancer (18; 86; 111; 159; 160) and blocking the Wnt pathway seems a quite promising target for cancer therapy.

4.4. Childhood cancers – Sarcomas

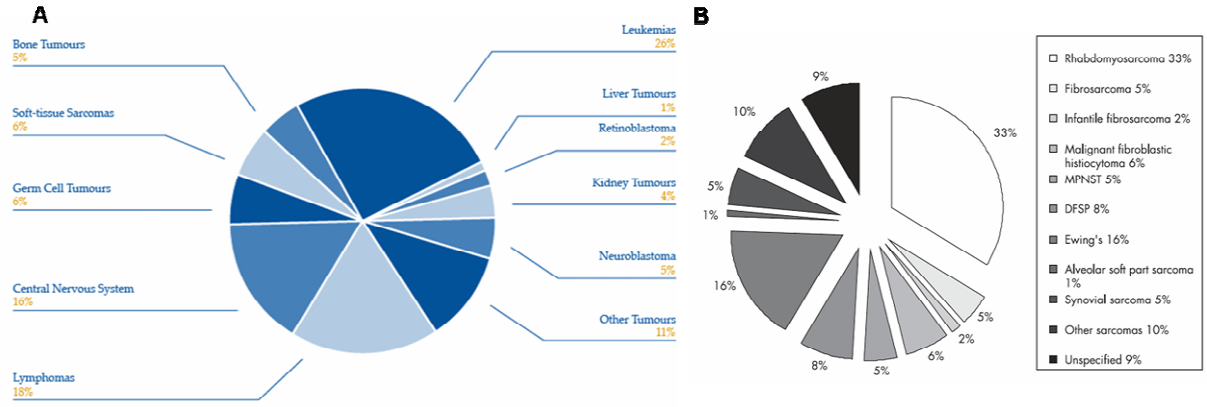

Figure 12: Distribution of childhood and adolescent tumors and Sarcomas
A) The most common childhood cancer is leukemia followed by brain tumors and sarcomas (161). B) Distribution of sarcomas in childhood (162)

Sarcomas are malignant solid neoplasms of mesenchymal origin arising from connective or supportive tissue such as muscle, bone, cartilage, fibrous and adipose tissue, blood and lymphatic vessels. Cancers originating from epithelial cells are named carcinomas and are prevalent in adults and rare in children (2%) showing a diverse spectrum of tumor types (Figure 12A) (163). The tumors can arise basically anywhere in the body. 1% of all tumors are sarcomas, nevertheless 20% of all soft tissue sarcomas are diagnosed under the age of 35 and 50% of bone sarcomas such as high grade Ewing's and osteosarcoma are detected in children and adolescents (15-29 years; Figure 12B). Furthermore, the incidence of sarcomas in children and adolescents is 10-fold higher compared to adults and after leukemia it is the third most common tumor type in childhood and adolescents (Figure 12A). Fourteen subtypes of sarcomas have been associated with tumor-specific chromosomal translocations which are involved in pathogenesis by generating novel fusion oncoproteins (164). In high grade Ewing's sarcoma, the EWS gene on chromosome 22 fuses with ETS-like oncogenes, most commonly FLI1 on chromosome 11 and drives constitutively expression of target genes. In the clinic, sarcomas are treated with chemotherapy, radiation therapy and surgery. Due to better understanding of the molecular mechanism hallmarking the tumor, combinatorial treatment approaches reduce tumor recurrence and thus, the mortality rate drastically dropped in the last three decades (165).

4.4.1. Cancer stem cells in sarcomas

Despite better treatment approaches and a better understanding of cancer, in some cases the tumor recurs. According to the CSC hypothesis, this may be due to a small subpopulation of cells with tumor initiating potential and self renewal properties which is resistant to commonly used chemotherapeutics (22; 24; 25). Hence, it is crucial to find new approaches targeting directly the CSC populations.

CSCs have not only been identified and characterized in carcinomas such as breast tumors (36), but also in several sarcomas. It is believed that mesenchymal stem cells (MSCs) are the origin of sarcomas (166; 167). However due to the hypothesis that CSCs can arise from a SC, a progenitor cell or a differentiated cell, several approaches were undertaken to identify the CSC subpopulation in sarcomas. First by trying to passage different sarcoma cell lines in serum-free medium with selected growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF) and platelet derived growth factor (PDGF) and then by characterizing drug resistance and expression of SC genes (e.g. oct4, nanog and sox2) of the so called sarcospheres (168). These genes are overexpressed in normal SC and progenitor cells and play an important role in embryogenesis and adult tissue repair.

Furthermore, the group of Hauptmann showed that combined high expression of SC genes like survivin, Hiwi and hTERT associates with a poor prognosis in soft-tissue cancer patients. High expression of all three genes was correlated with a significantly increased risk of tumor-related death (169; 170).

In addition, a $Nestin^+$ subpopulation was found in mesenchymal SCs. Nestin plays an important role in the development of the central and peripheral nerve system and is downregulated when precursors start to differentiate. After activation of mesenchymal SCs through epi- and genetic changes, this population gave rise to dermatofibrosarcoma being highly positive for Nestin (171).

Wilson et al. detected a subpopulation expressing high levels of embryonal SC genes such as Oct4, Nanog and Stat3 in canine osteosarcoma samples. Subsequently, a more resistant $CD117^+$/ $Stro1^+$ population was identified which is associated with metastasis (172; 173).

These investigations suggest that sarcomas are associated with the cancer stem cell model and it could be that they are organized hierarchically.

On that account, the most studied sarcoma in CSC research is the bone tumor Ewing's sarcoma (EWS). It is thought that the origin of CSCs is a mesenchymal SC. Additionally, the fusion protein EWS-FLI1 modulates the expression of SC genes of mesenchymal origin to-

wards EWS SCs (166; 174-176). CD133 is the marker describing a chemoresistant subpopulation in EWS leading to tumor formation after injection into immunocompromised mice (100; 177).

Furthermore, the group of Yeger identified a side population (SP) enriched under hypoxic conditions in neuroblastoma and alveolar rhabdomyosarcoma cells. The SP was also responsible for tumor development in xenograft models and expressed high levels of the SC gene oct4 (128).

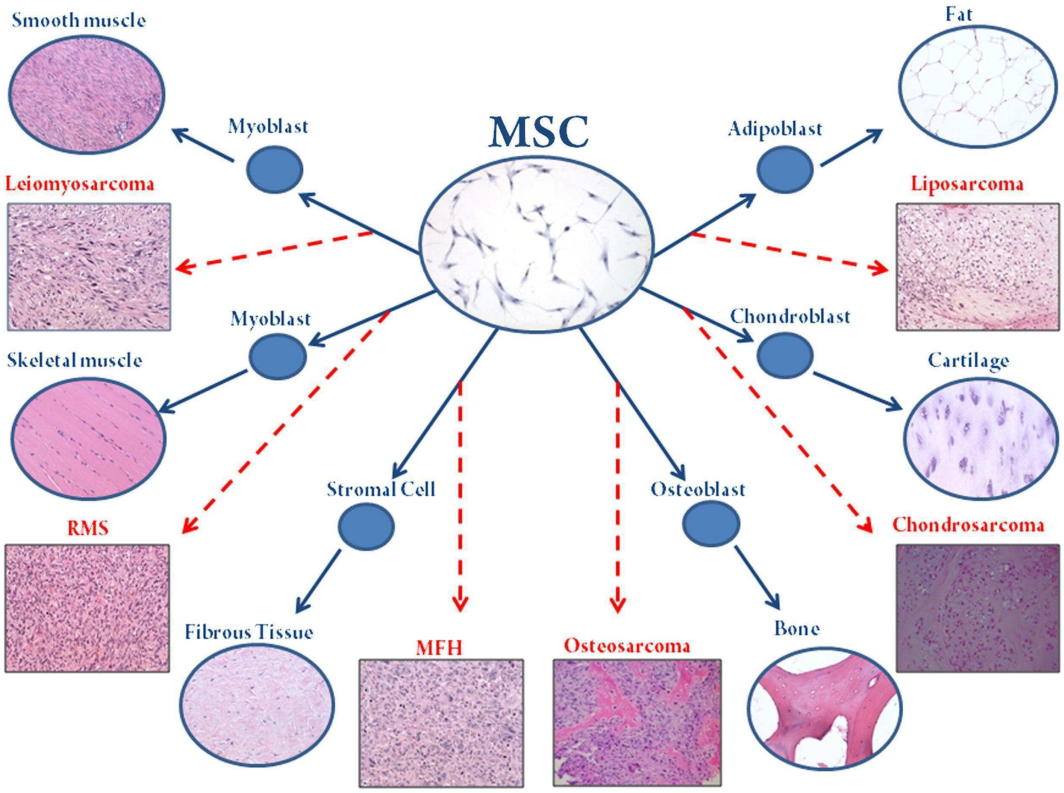

Figure 13: Accumulation of genetic lesions in mesenchymal stem cells leads to various sarcomas
MSCs differentiate into muscle, stroma, bone and fat cells, whereas mutations lead to abnormal growth and tumor formation such as RMS and osteosarcoma (167).

It was believed that the origin of RMS is a mesenchymal SC which changes into malignant cells after activation. In this regard, Langenau et al. developed a zebrafish model where they could show that knock-out of p53 leads to rhabdomyosarcoma formation and that the cell of origin is mesenchymal (178; 179). However, high expression levels of PAX3/7 was further associated with satellite cells being the precursors of muscle cells. Satellite cells, which are the adult muscle stem cells, and RMS are positive for PAX3/7 and the myogenic regulatory factors (MRFs) Myf5, myogenin and MyoD indicating that satellite cells could be as well the cell of origin for RMS (180-183). In contrast to these findings indicating a mesenchymal ori-

gin, Wachtel et al detected several neuronal genes upregulated in RMS patient samples and cell lines and the paired box proteins 3 and 7 also have an important role in neuronal development (181; 182; 184; 185). Hence, the cell of origin for sarcomas, especially RMS could be a mesenchymal SC (Figure 13), a satellite cell or a neuronal SC which differentiates into malignant muscle-like cell after activation (186).

Therefore, after identification of the fibroblast growth factor receptor 3 (FGFR3) as a CSC marker in embryonal RMS, Hirotsu et al. concluded that $FGFR3^{+}$ CSCs can be mesenchymal origin (187).

To date, it is not proven, whether sarcomas are organized in a hierarchical or in a stochastic manner (34; 35; 188). To do this, it would be necessary to first describe a subpopulation with self renewal properties *in vitro* and *in vivo* and then to characterize this subpopulation in detail.

4.4.2. Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common soft tissue cancer in childhood representing 4 to 8% of all pediatric malignancies (Figure 12B) (189; 190). RMS is one member of the small blue round cell tumors displaying various degrees of striated muscle differentiation and comprising of neuroblastoma, non-Hodgkin's lymphoma, Ewing's sarcoma, Wilm's tumor and RMS (191). Therefore, it is thought to originate from primitive mesenchymal stem cells (MSCs) (166; 167). RMS occurs in most parts of the body, but the most frequently found sites are spaces surrounding the brain and spinal cord and the limbs (Figure 14) (192). RMS is more common in childhood having a peak at 5 years of age and boys are reported to be more frequently affected than girls (1.4/1) (193). RMS is classified into different subtypes according to their histological features, expression of specific markers and molecular features such as chromosomal translocation (189). The most common subtype is embryonal RMS (eRMS, 60% of all RMS), whereas the most aggressive one is alveolar RMS (aRMS, 20% of all RMS). The other two subtypes, pleomorphic and botryoid RMS, are rare and are more common in adults.

RMS treatment is based on a multimodal approach involving surgery, chemotherapy and radiotherapy. The optimal timing and intensity of these treatment modalities must be planned with regard to prognostic factors such as site of occurrence and presence of metastasis at diagnosis and considering late effects of the treatment. Although the conventional treatment of RMS with chemo- and radiotherapy has been greatly improved (overall survival: aRMS~49%; eRMS~73%), the 5-year survival rate is still quite low in aRMS cases with me-

tastasis at diagnosis (10-30%), whereas the survival rate increases for localized forms of eRMS (93%) (189).

4.4.2.1. Embryonal Rhabdomyosarcoma (eRMS)

ERMS is the most common RMS subtype with a peak incidence in children younger than 6 years old. The prognosis is quite good due to its low metastatic potential, whereas eRMS cases in young adolescent are more aggressive and have a higher potential to invade into surrounding tissues. ERMS tumors are diagnosed histologically by staining the tumors for myogenic differentiation markers such as desmin and myogenin and by analyzing the morphology of the round or spindle-shaped tumor cells (Figure 14, down left). ERMS occurs mainly in the head and neck region and around the genitourinary track. Genetically, no rearrangements have been found in eRMS, whereas eRMS tumors are hyperdiploid with an increase in copy numbers for chromosomes 2, 7, 8, 12 and 13. Moreover, polymorphism studies revealed that eRMS is associated with loss of heterozygocity (LOH) on chromosome 11p15 (194) and 9q22 (30% of eRMS cases) (195). LOI on chromosome 11p15 and LOH of chromosome 9q22 are associated with increased expression of insulin-like growth factor 2 (IGF2) and decreased expression of Ptch1. Furthermore, the paired-box transcription factors PAX3/7 are highly expressed in eRMS samples (196).

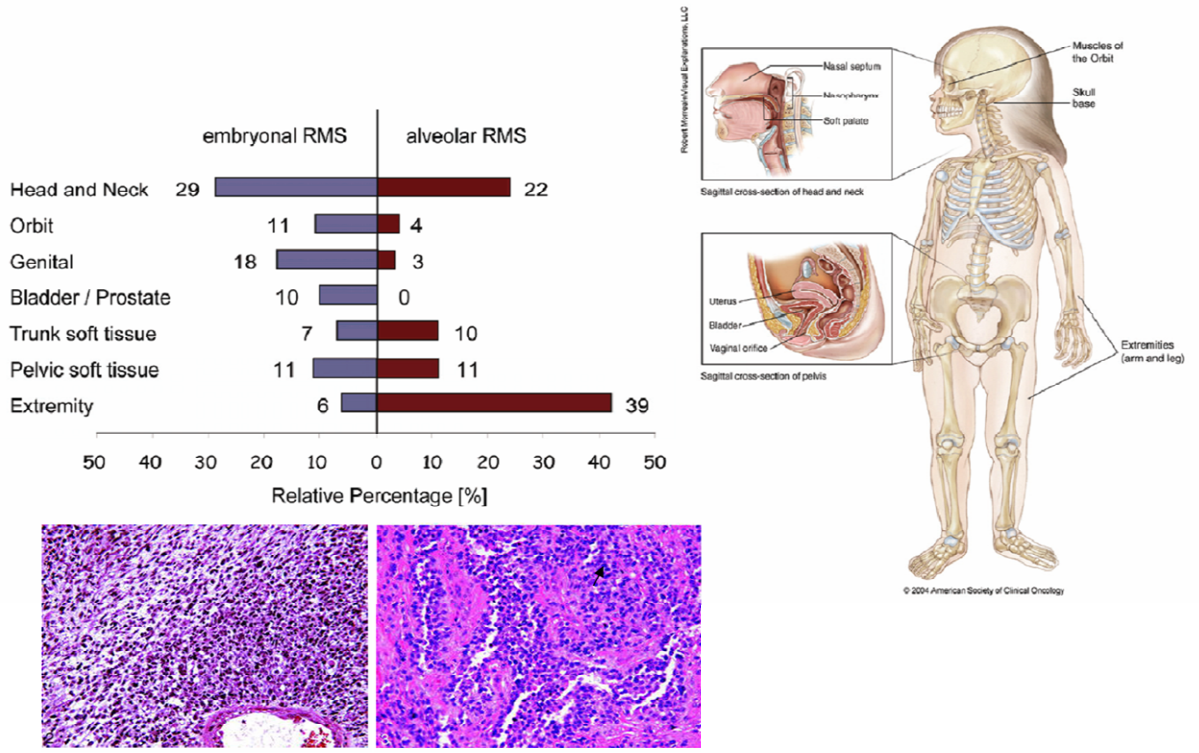

Figure 14: Rhabdomyosarcoma sites of occurrence, frequency in childhood and morphology
ERMS arises more in the head and neck region and in the genitourinary tract. The morphology of this subtype resembles more differentiated cells being positive for Myogenin and desmin (left side). ARMS are more de-differentiated resembling pulmonary alveoli separated by strands of fibrous tissue (arrow, right side). It occurs mostly in the extremities (192; 197).

4.4.2.2. Alveolar Rhabdomyosarcoma (aRMS)

The more aggressive subtype with high metastasizing potential is the less frequent one. It occurs in older children or adolescents in the trunk and extremities. The prognosis is poor with a 5-year survival rate of less than 50%. Characterization is based on histological features like round and loosely arranged cells resembling pulmonary alveoli separated by strands of fibrous tissue (Figure 14, down right) and molecular approaches such as fluorescence in situ hybridization (FISH) and real-time PCR to detect translocations. Most cases of aRMS are characterized by two pathogenomic translocations: t(2;13)(q35;q14) found in 55% and t(1;13)(p36;q14), found in 22% of the cases, both leading to the formation of fusion genes between the undisrupted PAX3 and PAX7 DNA binding domains, and the transactivation domain of the FKHR gene on chromosome 13. Normally, PAX proteins consist of a paired domain, an octapeptide linker, a homeodomain and a transactivation domain disrupted in the fusion proteins. The FKHR protein is disrupted in the DNA-binding domain (Figure 15).

Both fusion proteins PAX3/FKHR (P3F) and PAX7/FKHR (P7F) activate the target genes of PAX3 and PAX7 with a 10-100 fold higher potency than the corresponding wildtype PAX protein making them ideal targets for treatment (198-201). Moreover, they are used as diagnostic markers for aRMS and can be associated with a prognostic prediction (202). PAX3/FKHR fusion is associated with a highly metastatic phenotype and thus a poorer prognosis than PAX7/FKHR fusion (203). However, there are aRMS cases where no translocation was detected and 50% of the fusion negative cases could not be correlated with PAX3, PAX7 or FKHR. The meaning behind this finding is not clear yet, and more fusion negative cases should be analyzed (204).

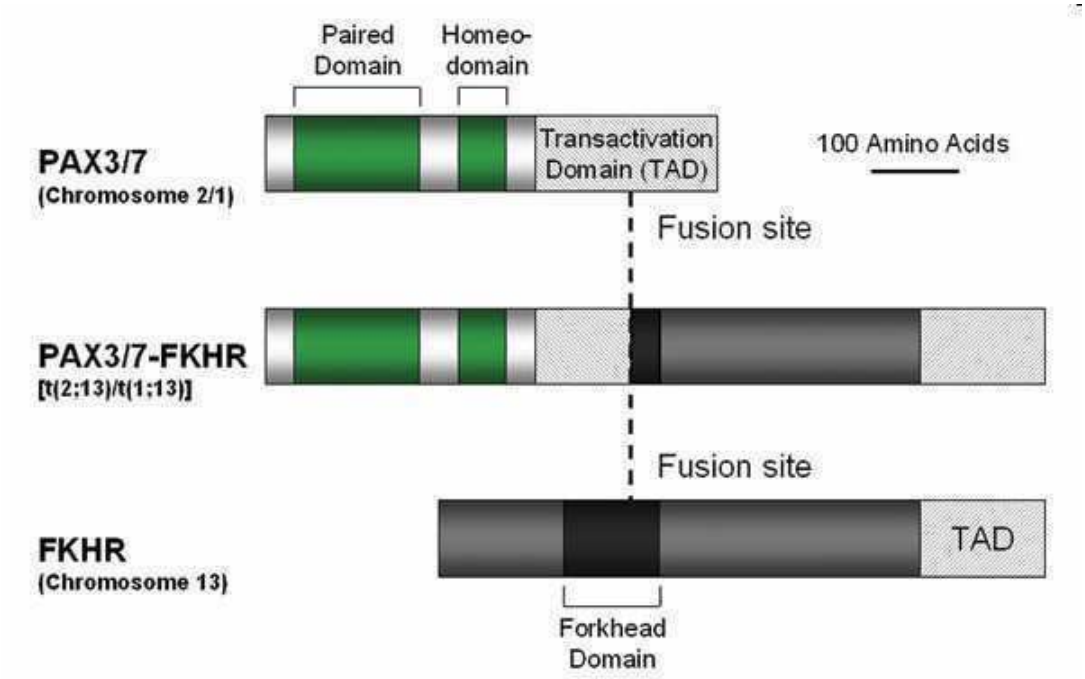

Figure 15: Schematic illustration of generation of the PAX3(7)/FKHR fusion protein
The DNA binding domain of PAX3 and PAX7 fuses with the transactivation domain of FKHR (205).

4.4.2.3. Aberrant signaling pathways

Diverse sets of mutations, translocations and amplifications are known in RMS. These alterations contribute to the formation and maintenance of RMS tumors. Mainly oncogenes and tumor suppressor genes are affected, such as p53, N-myc, ras, mdm2 and c-met, among others (206; 207). Furthermore, an autocrine growth factor loop is present in RMS leading to higher expression levels of several growth factors such as EGF, TGF-ß, IGF-I and VEGF. These growth factors activate constitutively specific growth factor receptor signaling cascades and thus favor tumor promoting effects like inhibition of differentiation, extensive pro-

liferation and evasion of apoptosis (208-211). Additionally, it was shown that an autocrine loop activates myostatin which negatively regulates muscle differentiation (212).

The fusion proteins are highly expressed in aRMS, but only P7F showed increased expression levels due to amplification, whereas P3F activity increases due to other mechanisms. Furthermore, it was shown that forced expression of P3F in fibroblast plus other events such as activation of N-myc, which is a direct target of P3F, mutations or alterations in p53 and ras and telomere stabilization result in oncogenesis *in vitro* and *in vivo*. Therefore, the translocation of PAX3 and FKHR leads to a fusion protein favoring several mechanisms such as increased proliferation, activated angiogenesis and muscle development. Furthermore, it inhibits simultaneously differentiation (212) and finally activates antiapoptotic pathways such as PI3K/AKT and MAPK/ERK pathway. Due to the LOH in eRMS and the loss of imprinting (LOI) in aRMS, IGF2 is highly expressed and IGF2 is also associated with the antiapoptotic pathways.

Furthermore, PAX3 regulates the transcription of c-met controlling cellular migration. In cancer, c-met is constitutively active which leads to extensive proliferation, migration, angiogenesis and less apoptosis. Therefore, permanent activation of c-met could lead to RMS (213) which was mentioned in several mouse models leading to RMS (214-216).

P3F activates directly the insulin-like growth factor receptor 1 (IGFR1) which is an antiapoptotic protein and targets of P3F such as AP2 are triggering the expression of IGF2, thus leading to an activation of the IGF pathway (207; 217).

Hence, many pathways triggering growth proliferation are constitutively activated in RMS through multiple para- and autocrine growth factor circuits.

5. Aim

In conclusion, despite the fact that the origin of rhabdomyosarcoma is still controversially discussed, the hypothesis that only a small subpopulation named cancer stem cells is responsible for tumor growth, resistance to therapy and recurrence, is appealing and specific targeting of this population for therapy is of great interest.

Thus overall aim of this thesis encompassed the identification and characterization of a cancer stem cell (CSC) population in RMS. In this regard the main questions were

1. Is there a TIC subpopulation with self renewal property in RMS?
2. Which markers are specifically expressed on the TICs?
3. Can it be correlated with clinical data?

Aim1: For the identification of TICs, we tried to generate sphere cultures *in vitro* being enriched in a subpopulation with self renewal property. To investigate the tumorigenic potential of these spheres, limited dilutions *in vivo* are indispensable. We performed differentiation assays to investigate the differentiation potential of the sphere cultures. With these assays we aimed to identify and enrich a subpopulation being more tumorigenic, differentiate into various lineages and with self renewal property.

Aim2: We performed an exon microarray where we compared different passages of the sphere cultures with adherent cells to investigate whether a signature change is occurring and to identify genes, upregulated or downregulated, encoding membrane proteins. Furthermore, this approach could give us more information about the signaling pathways active or inactive in the subpopulation.

Aim3: Finally, we would like to evaluate the obtained results on primary material and correlate it with clinical data.

6. Results

6.1 Rhabdospheres enrich a CD133 positive potential rhabdomyosarcoma stem cell population

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Carole Gengler: Figure 1e; Figure 4c

Holger Moch: Financial support

Felix K. Niggli: Financial support

Beat W. Schäfer: Principal Investigator; Conception; Discussion and Writing

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CD133 positive Rhabdomyosarcoma stem-like cell population is enriched in Rhabdospheres

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Abstract

Cancer stem cells (CSCs) have been identified in a number of solid tumors, but not yet in Rhabdomyosarcoma (RMS), the most frequently occurring soft tissue tumor in childhood. Hence, the aim of this study was to identify and characterize a CSC population in RMS using a functional approach.

We found that RMS cell lines can form rhabdospheres in stem cell medium containing defined growth factors over several passages. Using an orthotopic xenograft model, we demonstrate that a 100 fold less sphere cells result in faster tumor growth compared to the adherent population suggesting that CSCs were enriched in the sphere population. Furthermore, stem cell genes such as *oct4*, *nanog*, *c-myc*, *pax3* and *sox2* are significantly upregulated in rhabdospheres which can be differentiated into multiple lineages such as adipocytes, myocytes and neuronal cells.

Surprisingly, gene expression profiles indicate that rhabdospheres show more similarities with neuronal than with hematopoietic or mesenchymal stem cells. Analysis of these profiles identified the known CSC marker CD133 as one of the genes upregulated in rhabdospheres, both on RNA and protein levels. $CD133^{+}$ sorted cells were subsequently shown to be more tumorigenic and more resistant to commonly used chemotherapeutics. Using a tissue microarray (TMA) of eRMS patients, we found that high expression of CD133 correlates with poor overall survival. Hence, CD133 could be a prognostic marker for eRMS.

These experiments indicate that a $CD133^{+}$ CSC population can be enriched from RMS which might help to develop novel targeted therapies against this pediatric tumor.

Introduction

The cancer stem cell hypothesis suggests that a small subpopulation of cells sharing common characteristics with normal stem cells (SCs) - such as capacity to self renew, potential to differentiate, extensive proliferation *in vivo*, and resistance to chemotherapeutics - is responsible for tumor development [1] and that tumors are organized hierarchically. This concept was first established in acute myeloid leukemia (AML) [2] and subsequently also in a number of solid tumors such as breast cancer where a $CD44^{+}/CD24^{-/low}$ CSC population was identified [3], in brain tumors [4], colon cancer [5], and melanomas [6]. Additionally, CSCs are postulated to be more resistant to standard chemotherapy [7; 8; 9; 10; 11] and might be responsible for tumor recurrence usually observed in the clinics. However, the concept is still controversial and the frequency of CSCs might vary between tumor entities. Some tumors

might not be hierarchically organized at all. Therefore, the existence of such a cellular subpopulation most likely has to be established for each tumor type [12].

Rhabdomyosarcoma (RMS) is the most common soft tissue tumor in childhood representing 5 to 8% of all pediatric malignancies [13]. RMS is a member of the small blue round cell tumors additionally comprised of neuroblastoma, non-Hodgkin's lymphoma, Ewing's sarcoma and Wilm's tumor [14]. It occurs in most parts of the body, but more frequent sites are spaces surrounding the brain, the trunk and genitourinary tract [15]. It has been suggested that mesenchymal stem cells (MSCs) might be the origin of rhabdomyosarcomas and accordingly the origin of a potential rhabomyosarcoma stem cell might also be a mesenchymal one [16; 17]. However, some reports indicate that also neuronal cells can transform into malignant myogenic cells after activation and a large number of neuronal genes are expressed in RMS. Hence the origin of potential RMS stem cells remains to be determined [18; 19].

CD133, also known as Prominin1, is a five transmembrane protein with eight potential N-glycosylation sites. It was first described in murine neuroepithelial cells and was recognized as a human hematopoietic SC marker, because hematopoietic $CD34^+$ progenitor cells express CD133 [20]. CD133 has been suggested as CSC marker in brain tumors [21], breast [3], colon [22], pancreatic [23], liver [24], skin [25], prostate cancers [26] and Ewing's sarcoma [27]. Furthermore, $CD133^+$ glioma stem cells are more resistant to chemotherapy and radiation than bulk and the CD133 negative population [8]. Moreover, CD133 downregulation induced differentiation in neuroblastoma cell lines and thus increased sensitivity to drug treatment [28]. Therefore, CD133 could by itself also represent a potential marker for targeted therapy. Nevertheless, CD133 positive CSC populations in melanoma and prostate cancer are still controversially discussed [29; 30].

Here, we enriched for a CSC population in rhabdosphere cultures which are 100 fold more tumorigenic than adherent cells in xenograft experiments. This subpopulation expressed the stem cell genes *sox2*, *oct4*, *nanog*, *c-myc* and *pax3* to significantly higher levels and retains the capability to differentiate into adipocytes, myocytes and neuronal cells. Furthermore, the known stem cell marker CD133 was upregulated in rhabdospheres. $CD133^+$ cells characterize a subpopulation which is more tumorigenic and resistant to chemotherapy than the negative population. In addition, high CD133 expression in human RMS samples correlated with a poor overall survival.

Thus, our study demonstrates for the first time that rhabdospheres can be formed from RMS cells which are enriched in a $CD133^+$ CSC population.

Material and Methods

Cell culture methods

Rh36 and Rh18 (kindly provided by Peter Houghton (St Jude Children's Hospital, Memphis, TN, USA)), RD, U87MG and MRC5 (purchased from the American Type Culture collection (LGC Promochem, Molsheim Cedex, France)) and Ruch2 (established in house) were cultured in Dulbecco's modified Eagle medium containing 10% FCS.

Sphere cultures were derived from and enriched over several passages by seeding the cell lines in a defined serum free medium (SC medium) consisting of Neurobasal medium (Invitrogen) supplemented with 10ng/ml EGF (R&D Systems), 20ng/ml b-FGF (R&D Systems) and 2x B27 (10ml; Invitrogen) [31]. Adipogenesis was induced as described [32; 33]. Briefly, after preparing spheroids, cells were seeded into chamber slides and treated with or without 0.1% DMSO for 3 days. After 8 days in differentiation medium, containing 85nM insulin, 2nM triiodthyronine (T_3) and 10 % FCS, cells were stained with Oil-RedO (ThermoScientific) [34]. Neurogenesis and Myogenesis were assayed as described [35]. Briefly, cells were seeded into 6 well plates and treated with different concentrations of retinoic acid RA (1nM, 10nM, 300nM). After 24 days in differentiation medium containing RA and 0.5% FCS, cells were fixed in 4% PFA and stained for differentiation markers. Resistance to chemotherapeutics was tested by seeding 2000 cells in a 6-well plate 48 hours before treatment. The cells were treated twice a week with different concentrations of cisPlatin (Sigma; 10µM and 50µM) and Chlorambucil (Sigma; 6.45µM). Twice a week, colonies were counted and documented. For visualizing the colonies, we stained them with crystal violet according to Franken, et al. [36].

Immunofluorescence, immunohistochemistry and flow cytometry/sorting

For immunofluorescence staining, cells were fixed in 4% PFA and blocked in medium containing 10% FCS and 0.5% Triton. Cells were stained over night at 4°C for CD133 (1/100) (polyclonal antibody, Abcam), GFAP (1/300) (monoclonal antibody, R&D Systems), myogenin (1/2) (F5D; monoclonal antibody, Developmental Studies Hybridoma bank) and N-CAM (1/2) (5.1H11; Developmental Studies Hybridoma bank)). Alexa Fluor 488 or 594 (1/200) (Invitrogen) antibodies were used as secondary antibodies. All stainings were analyzed with an Axioskop2 mot plus fluorescence microscope (Zeiss). Xenograft tumors were embedded in paraffin, fixed and analyzed for H&E, Myogenin (1/20) (Myf4, monoclonal antibody, Novocastra Laboratories Ltd) and desmin (1/20) (monoclonal antibody; Dako) by

immunohistochemistry. As secondary antibody a HRP labeled rabbit anti-mouse antibody (Epitomics) was used. Stainings were visualized with the Refine DAB-Kit (Leica).

For flow cytometry, cells were trypsinized, washed and stained (1/10) with a fluorochrome labeled antibody (CD133/2-APC, Miltenyi). All samples were measured with a BDFACSCanto II flow cytometer (BD Bioscience) or MoFlo high speed cell sorter (DakoCytomation) and analyzed with the software FlowJo.

Molecular methods

RNA was extracted using RNeasy Plus Mini Kits (Qiagen). Reverse transcription was carried out using the high-capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. RNA and cDNA concentrations were measured with a Nanodrop ND1000 spectrometer. Quantitative Real-Time PCR was performed using validated TaqMan Gene Expression Assays (Applied Biosystems) for POU5F1/OCT3-4 (Hs02397400_g1), NANOG (Hs02387400_g1), SOX2 (Hs01053049_s1), CMYC (Hs00153408_m1), PAX3 (Hs00992437_m1), NMYC (Hs00232074_m1), PROM1/CD133 (Hs01009261_m1) and GAPDH (Hs99999905_m1) as an endogenous housekeeping gene for normalization. Reactions were run using the standard conditions on an ABI 7900HT Fast Real-Time PCR machine. Relative fold difference was calculated using the -ΔΔCt method. Gene expression profiling of different RNA samples from different sphere passages (early = passage 3; intermediate = passage 5 to 7; late = passage 10) and their corresponding adherent control was performed by an Affymetrix Exonmicroarray (HuEx-1_0-st-v2). The samples were analyzed with the Genespring10 and Ingenuity IPA software and compared with published data sets (hematopoietic (GSE2666), FM95 (GSE10435), embryonic skeletal myoblast (GSE3230), mesenchymal stem cells (GSE2248), embryonic stem cells (GSE9440), neuronal cells (GSE10691), glioblastoma cells and patient samples (GSE7181), neurospheres (GSE8049) and prostate cancer samples (GSE10832)). The correlation of the samples was analyzed with a script programmed in R (Functional Genomic Center Zurich).

Xenograft experiments

Xenograft experiments were approved by the veterinary office of the Canton of Zurich.

Different amounts of adherent cells and their corresponding sphere cultures were injected intra muscularly into the right leg of NOD.CB17-*Prkdc*^{scid}/J (NOD/Scid) and NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG)

mice (The Jackson Laboratory) and tumor size was determined every 2 to 3 days by measuring two diameters (d_1 and d_2) in right angles of both legs with a calliper. Tumor volumes were calculated using the following formula $V= [4/3\ \pi\ ½(d_1+d_2)]_{right\ leg} - [4/3\ \pi\ ½(d_1+d_2)]_{left\ leg}$.

Statistical analysis

For in vitro experiments, Student's t test was used on triplicates. P values of less than 0.05 were considered significant.

Results

Rhabdospheres are enriched with cancer stem-like cells

To determine whether RMS cells might contain a subpopulation of CSC cells, we attempted to grow embryonal rhabdomyosarcoma (eRMS) cell lines (RD, Rh36, Ruch2 and Rh18) as rhabdomyosarcoma spheres (rhabdospheres) in stem cell medium (SC-medium). A glioblastoma cell line (U87MG) and fibroblast cells (MRC5) were used as positive and negative controls, respectively. Three eRMS cell lines (RD, Rh36 and Ruch2) formed rhabdospheres under these conditions over several passages (Figure 1A). To test, whether sphere cells could be serially enriched, we seeded 20000 sphere cells over several passages into the SC-medium and determined the number of spheres at each passage (Figure 1B). Compared to the positive control U87MG sphere cultures which showed the highest enrichment over 10 passages (up to 1750 spheres per 20000 cells), 1300 spheres were counted for RD cultures after 10 passages, while Ruch2 and Rh36 sphere cultures could also be enriched albeit to a lesser extent (600 counted spheres) indicating that a subpopulation of cells with self renewal property can be enriched from three different eRMS cell lines. To investigate whether this self renewing subpopulation is more tumorigenic than the adherent population, we injected different numbers of RD cells and their corresponding sphere cultures (10^6, 10^5 and 10^4) intramuscularly (i.m.) into the right leg of NOD/Scid mice (n=6) and measured tumor growth over several weeks (Figure 1C). Xenograft tumors from sphere cultures started to grow around day 40 after injection, compared to adherent cells where we detected the earliest tumor growth around day 80 post injection. Moreover, tumor growth was observed when we injected 100 fold less sphere cells (10^4), whereas no tumor growth was seen using the same number of adherent cells. 125 days after injection, in two out of six NOD/Scid mice injected with 10^5 adherent cells, a small tumor was seen, while we detected tumors in every mouse injected with 10^5 sphere cells already after 60 days. These results were subsequently confirmed in a second

mouse strain, namely NSG mice, where tumor growth was observed with 10^5, 10^4 and 10^3 (one out of three mice) injected sphere cells, but only with 10^6 adherent cells (Figure 1D). Therefore, in both mouse models cells from sphere cultures are more tumorigenic and fewer cells are needed for tumor growth compared to adherent cells. To demonstrate that all xenograft tumors were indeed RMS tumors, we collected tumor samples and constructed a xenograft tissue microarray (TMA) with adherent RD cells and corresponding sphere cultures as controls. Stainings of the TMA with RMS markers, myogenin and desmin, was positive in both adherent cells and spheres (Figure 1E) which were negative for markers of other small blue round cell tumors (CD45, CD99, cytokeratin (CK), S100b, Synaptosin, smooth muscle actin (SMA) and WT1; data not shown). Furthermore, all xenograft tumors displayed typical RMS hallmarks such as multinucleated cells and positive stainings for desmin and myogenin, irrespective of the mouse strain they were grown in. These results confirmed that all xenograft tumors represented RMS tumors with similar features. We conclude from these experiments that a subpopulation of RMS cells can be enriched in sphere cultures over several passages which is more tumorigenic *in vivo* and therefore could represent a potential CSC population.

Sphere cultures have stem cell characteristics

To further substantiate the notion that rhabdospheres are enriched for CSC, we quantified the expression levels of several known SC genes like *oct4*, *nanog*, *c-myc*, *sox2* and *pax3* with real-time PCR in different passages of sphere cultures (passages 3, 7, 10) compared to adherent cells. While *oct4* and *pax3* showed the highest upregulation in RD sphere cultures ($P< 0.0001$), also *c-myc* ($P=0.0016$), *sox2* ($P= 0.0068$) and *nanog* ($P=0.0028$) were significantly upregulated (Figure 2A). Similar results were obtained with Rh36 cells with the exception of *pax3* and *c-myc* which did not change significantly (Figure 2B). This could be due to already high endogenous expression levels in the adherent Rh36 cell line when compared to RD adherent cells (data not shown). Therefore, we selected RD cells for all subsequent experiments.

It has been shown that cells with multilineage differentiation potential can differentiate into neuronal cells, myocytes and adipocytes after treatment with dimethylsulfoxid (DMSO) or retinoic acid (RA) [32; 35; 37; 38]. On that basis, we next assessed to which extent RD cells can be differentiated towards these lineages. First, we treated adherent and sphere cultures with different concentrations of RA (1nM, 10nM and 300nM). After 24 days, cells were stained with myogenic (myogenin, N-CAM) and neuronal markers (GFAP, N-CAM) (Figure 2C, D). Although adherent cells expressed low levels of

myogenin (3,6% and 11,5%) after treatment (Figure 2C), sphere cultures showed much stronger upregulation of myogenin positivity (~ 50%) (Figure 2D). The highest expression of N-CAM (52%) was detectable after treatment with 10nM RA. While both spheres and adherent cells were negative for myogenin when treated with 300nM RA, we observed positive stainings for N-CAM and GFAP (9,4%), indicative of neuronal differentiation, only in sphere cultures (Figure 2D) and not in adherent cells (Figure 2C). In contrast, no GFAP positive cell was found after 1 and 10nM RA treatment (data not shown). Untreated controls were negative for all markers analyzed (data not shown).

To differentiate cells towards adipocytes, we treated spheroids from both adherent and sphere cells for 3 days with DMSO. After subsequent cultivation in appropriate differentiation medium for 8 days, around 5% of DMSO treated adherent cells were positive for fatty vacuoles (Figure 2E). However, sphere cultures had positively stained fatty vacuoles in up to 90% (mean 73.75%) of the cells when treated with DMSO (Figure 2E).

In conclusion, sphere cultures had a significantly increased expression level of stem cell genes and regained the capability to differentiate towards neurogenic, myogenic and adipogenic lineages with appropriate stimulants. These results indicate that stem-like cells are enriched in rhabdospheres.

CD133 is upregulated in sphere cultures

To characterize sphere cultures in further detail and to identify marker proteins specifically up- or downregulated, a gene expression profiling was performed with a human exonmicroarray (HuEx-1_0-st-v2) for both RD and Rh36 cells and three different passages (early, intermediate and late) of their corresponding spheres.

In Figure 3A, a heat map of all samples is shown which revealed that RD and Rh36 cells cluster with their corresponding sphere cells indicating that both cell lines are more different from each other than their different passages. Nevertheless, in total 2217 genes (upregulated 1568 genes, downregulated 649 genes) are differentially expressed in RD spheres compared to adherent cells with a fold change of at least two. To restrict the number of genes and to find potential markers characterizing the rhabdospheres, a metaanalysis with different microarray samples publicly available (hematopoietic, FM95, embryonic skeletal myoblast, mesenchymal stem cells, embryonic stem cells, neuronal cells, glioblastoma cells and patient samples, neurospheres and prostate cancer samples) was implemented. All RMS samples, both adherent and rhabdospheres (red (RD) and pink (Rh36)), clustered together with neuronal and glioblastoma cells and their spheres, and patient samples (depicted in green) (Figure

3B). Due to this observation, we searched for genes commonly up- or downregulated in RD and glioblastoma sphere cultures compared to their corresponding adherent cells with a fold change of at least two (Table 1). 31 genes were identified and further subgrouped according to their subcellular localization; membrane (8 genes), secreted (1 gene), endoplasmatic reticulum ER membrane (1 gene), golgi apparatus (1 gene), cytoplasm (12 genes) and nucleus (8 genes). In addition, 12 genes are commonly downregulated (membrane (6), secreted (2), cytoplasm (4)). To be able to identify and isolate a putative CSC population, we were interested mainly in membrane proteins of which we identified 14 genes. One obvious candidate gene in this list was CD133 or Prominin1 which is a well described SC and CSC marker. Therefore, we validated CD133 as a potential marker of rhabdospheres at the expression level by performing real-time PCR (Figure 3C). In sphere cultures of both RD and Rh36, CD133 expression was indeed significantly upregulated. To verify these results on protein level, RD cells and spheres were stained for CD133 and analyzed by flow cytometry (Figure 3D), fluorescence microscopy (Figure 3E) and western blotting (Figure 3F). In all experiments, CD133 is upregulated in rhabdospheres compared to adherent cells also on protein level.

These experiments suggest that $CD133^+$ cells , a known CSC marker, are enriched in rhabdospheres and CD133 might be a potential CSC marker in RMS.

$CD133^+$ RMS cells are more chemoresistant and tumorigenic

To verify whether a $CD133^+$ subpopulation is more tumorigenic and resistant to commonly used chemotherapeutics in RMS, we sorted RD cells for CD133 positive and negative ($CD133^+$, $CD133^-$) populations (Figure 4A) and performed limiting dilutions by orthotopical injections into NOD/Scid mice using adherent RD cells and unsorted bulk RD cells as controls. In contrast to the control where the highest number of cells injected (10^6 cells) developed a tumor, mice injected with $CD133^-$ cells (10^5 – 10^2) did not develop any tumor after 140 days. In contrast, we could detect at least one tumor in the $CD133^+$ injected mice in three out of four dilutions (Figure 4B). To demonstrate that these tumors are indeed RMS tumors, we analyzed them by immunohistochemistry using known RMS markers as described before. All tumors were positive for desmin and myogenin and histologically identical with RMS tumors (Figure 4C). To investigate potential resistance to commonly used chemotherapeutics, we seeded sorted cells at low density 48 hours before starting treatment with cisPlatin and Chlorambucil. Cells were treated twice a week and colonies obtained were counted after staining with crystal violet

(Figure 4D). $CD133^+$ sorted RD cells were more resistant to treatment and formed viable colonies which developed significantly less in the $CD133^-$ population.

Therefore, rhabdospheres are enriched for a $CD133^+$ population being more tumorigenic and resistant to cisPlatin and Chlorambucil.

High expression of CD133 correlates with poor overall survival

Finally, we investigated whether a $CD133^+$ subpopulation is also present in human patient material. To this end, we stained a human RMS TMA, first described by Wachtel [19], for CD133. For quantification, we scored for two variables, namely intensity of staining and number of positive cells. The added scores were used to classify the tumors as having negative, low, middle or high expression. ERMS patients showing no or low to intermediate CD133 expression showed an overall survival around 75% which is comparable with the survival rate of translocation negative RMS patients [39]. In contrast, patients with high expression of CD133 had a clearly worse survival (less than 50%, p=0.0272, Figure 5A). Representative tumor sections of high, intermediate and low CD133 stainings are shown in Figure 5B.

These results therefore indicate that CD133 is a potential CSC marker in eRMS that might identify eRMS patients with a poor outcome.

Discussion

Due to a better understanding of tumor organization, new treatment approaches that target directly a CSC population now seem possible [22]. It has been reported that not only leukemia [2] and carcinomas [3] have a subpopulation of cells with self renewal properties [27], but also some sarcomas such as Ewing's sarcoma might follow the cancer stem cell model [1]. For the most common sarcoma in childhood, RMS, no clear subpopulation has been identified until now [16; 17]. Therefore, we used a functional approach to investigate whether rhabdomyosarcoma tumors might have a subpopulation enriched in CSCs and are hierarchically organized.

We first adopted a sphere forming assay to enrich a subpopulation with stem cell properties *in vitro*. Testing different conditions of growth factor concentrations and media, sphere formation over several passages could be observed only in one condition which was described previously as a neuronal stem cell medium [31]. Several lines of evidence then indicate that these rhabdospheres are enriched for stem-like cells. First, limiting dilution in two different immunosuppressed mouse strains indicate that

the rhabdosphere population is at least 100 fold more tumorigenic than adherent cells. In contrast, culturing cell lines representing the alveolar subtype of RMS in the same stem cell medium leads to formation of rhabdospheres which surprisingly were not tumorigenic after injection into immunosuppressed mice (data not shown). Hence, it seems unlikely that media conditions themselves were responsible for induction of the observed phenotypes and rather selection of a preexisting subpopulation was occurring specifically in eRMS. Second, our data analyzing expression levels of stem cell genes in rhabdospheres compared to adherent cells demonstrate that the stem cell genes *oct4, nanog, sox2*, *c-myc* as well as *pax3*, are significantly upregulated. While *sox2*, *nanog*, and *oct4* are required for induction of the pluripotent stem cell phenotype, *c-myc* expression also correlates with tumor formation and upregulation of this oncogene could trigger the higher tumor initiating potential [40]. *Pax3* is a known developmental marker expressed during muscle and brain development, repressed in adult tissue and connected to tumor formation and a poor overall survival [41; 42; 43].

As an additional hallmark of cancer stem cells [1], we investigated whether rhabdospheres have the potential to differentiate into multiple lineages. Indeed, rhabdospheres treated with DMSO and RA, respectively, differentiate towards adipogenic, myogenic and neurogenic lineages similar to what has been observed in cells with multilineage differentiation potential such as embryonal carcinoma cells [32; 33; 35]. These data support the concept that rhabdospheres contain cells with stem-like features and that RMS tumors are hierarchical organized [1].

Previous studies have suggested that mesenchymal stem cells could be the origin of RMS [17; 44; 45]. In contrast, our metaanalysis of exon microarray data with published data sets revealed that RMS samples had an expression profile more similar to neuronal cells and patients than mesenchymal stem cells. Furthermore, expression profiles also detected a large number of neuronal genes being expressed in RMS biopsies [19; 46; 47; 48] such as *pax3* which is crucial for the development of both the myogenic and neuronal lineage [42]. Interestingly, it has been demonstrated earlier that a population of myogenic, myf-5 positive cells can be derived from neural tube during mouse development [49]. These myf-5 positive cells co-express both neuronal and muscle markers, raising the intriguing possibility that the cell of origin of our CSC population could also be a multipotential stem cell derived from cells in the neuronal compartment. In support of this, it has also been described that neuronal stem cells can differentiate into malignant muscle cells after activation [18]. However, this issue needs to be addressed further in the future.

Previous studies have shown that CD133 marks hematopoietic stem cells [20] and cancer stem cells [29], in particular neuronal and mesenchymal CSCs [21]. Moreover, a $CD133^+$ population was identified as a CSC population in sarcomas such as Ewing's sarcoma [27] and osteosarcoma [50] which was more resistant to chemotherapy and radiation [7; 8; 9; 10; 11]. It was therefore not surprising that CD133 emerged as a marker for RMS CSC in our study as well. Interestingly, also the fraction of $CD133^+$ cells in both Ewing's sarcoma and RMS seem to be similar. It has been reported that expression of FGFR3 might mark a tumorigenic subpopulation in RMS. However, we did not find an increase in mRNA expression of this receptor in rhabdospheres (data not shown). The same report also found that CD133 positive cells were not more tumorigenic than the negative population. However, the discrepancy with our study might be explained by the different CD133 epitopes that were used in the two studies. Here, using CD133 as a marker to sort cells which were then injected orthotopically into mice without prior cultivation in stem cell media, we readily detected tumor growth at lower cell numbers in CD133 positive versus CD133 negative cells. Indeed, in the $CD133^+$ injected group one mouse at every dilution developed a RMS tumor which was not observed in the $CD133^-$ population. The relatively low tumorigenicity detected in the sorted population in general is likely due to impaired viability of the cells by the sorting procedure. Interestingly, $CD133^+$ sorted cells were also more resistant to cis-Platin and Chlorambucil treatment suggesting that anti-apoptotic or mismatch repair proteins are active. Indeed, we observed upregulation of several transcripts encoding mismatch repair proteins in rhabdospheres (data not shown).

Finally, we stained a human RMS tissue microarray (TMA) [19] for CD133 to demonstrate that a $CD133^+$ population is also present in human tumor biopsies. Patients with high positivity for CD133 were found to have the worst overall survival, which could be explained by a higher recurrence. However, in a multivariate analysis using a cox regression model, we were not able to demonstrate that CD133 is an independent prognostic marker for eRMS since the number of patients in this group was too low. More patients will have to be included therefore in a future study. Nevertheless, CD133 might represent the first candidate marker to identify eRMS patients with poor survival and might be used to stratify patients in the future.

Conclusion

Overall, our results demonstrate that cells with self renewal property that can drive tumorigenicity and have the potential to differentiate into multiple lineages are enriched in rhabdospheres. With CD133,

we identified an already known CSC marker in an additional sarcoma [27; 50] whose expression also correlated with a poor prognosis in eRMS patients. Further characterization of this CD133 positive CSC population might lead to a better understanding of the development of RMS. It now seems possible to screen directly for therapeutically active substances targeting the CSC subpopulation in eRMS to further advance treatment of this childhood sarcoma.

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Table 1: List of genes up- or downregulated at least two fold in RD rhabdospheres

upregulated			downregulated	
Localization	**Chosen Gene IDs**	**Gene Symbol**	**Chosen Gene IDs**	**Gene Symbol**
Membrane	2535	FZD2	2674	GFRA1
	7976	FZD3	3778	KCNMA1
	8842	PROM1	4907	NT5E
	23554	TSPAN12	7010	TEK
	51678	MPP6	7057	THBS1
	55704	CCDC88A	23768	FLRT2
	57633	LRRN1		
	84216	TMEM117		
Secreted	255743	NPNT	4015	LOX
			7424	VEGFC
Golgi apparatus ER membrane	22836	RHOBTB3		
	80055	PGAP1		
Cytoplasm	2037	EPB41L2	3433	IFIT2
	3157	HMGCS1	3437	IFIT3
	4133	MAP2	9060	PAPSS2
	6860	SYT4	10231	RCAN2
	9315	C5orf13		
	9456	HOMER1		
	9735	KNTC1		
	54874	FNBP1L		
	55792	PCID2		
	56992	KIF15		
	91057	CCDC34		
	113263	GLCCI1		
Nucleus	7552	ZNF711		
	9735	KNTC1		
	10926	DBF4		
	55769	ZNF83		
	64105	CENPK		
	81931	ZNF93		
	84250	ANKRD32		
	90317	ZNF616		
	151648	SGOL1		

Figure legends

Figure 1. Cancer stem-like cells are enriched in Rhabdospheres

A), B) Embryonal rhabdomyosarcoma (eRMS) cell lines (RD, Rh36 and Ruch2) were cultured in stem cell medium (SC-medium) over several passages. A glioblastoma (U87MG) and a fibroblast (MRC5) cell line were used as controls. A) Representative phase contrast pictures of cultured RD, Ruch2 and Rh36 sphere cultures (400x magnification). B) Subpopulation enrichment over several passages (x-axis) was estimated by counting the obtained spheres per cell (y-axis). C), D) Limited dilution (10^6, 10^5 and 10^4) of adherent versus sphere cells *in vivo*. Cells were intramuscularly (i.m.) injected into NOD/Scid (n=6) (C) and NSG mice (n=3) (D) at the indicated numbers and tumor growth (y-axis; tumor volume in mm^3) was measured over time (x-axis). E) Immunohistochemical (IHC) stainings of xenograft tumor sections on a xenograft tissue microarray (TMA). Adherent and sphere cells were used as controls on the TMA. The TMA was stained for H&E and RMS markers (desmin and myogenin). Representative IHC stainings are shown (400x magnification). The small inserts represent magnifications of positively stained cells.

Figure 2. Sphere cultures have stem cell characteristics

A), B) Expression analysis of stem cell genes (*oct4*, *nanog*, *sox2*, *c-myc* and *pax3*) by Real-time PCR. RD (A) and Rh36 (B) adherent cells and 3 different sphere culture passages (3x, 7x, 10x) were compared. C), D) RD cells (C) and their corresponding sphere cultures (D) were treated with retinoic acid (1nM, 10nM, 300nM) for 24 days and stained for differentiation markers (N-CAM, myogenin and GFAP). Percentage of positivity was calculated by counting 3 different random microscopic fields with at least 30 cells. E) RD cells and spheres were treated with 0.1% DMSO for 3 days. After additional 8 days, cells were stained for OilRedO. Percentage of cells with fatty vacuoles was calculated by counting 4 independent slides. Representative pictures and magnifications (small box) of OilRedO stainings are shown. For A) ★ ★ ★ P<0.0001; ★ ★ P=0.0028; ★ ★ P=0.0016; ★ ★ P=0.0068. For B) ★ ★ P=0.0015; ★ P=0.0281; ★ P=0.0275. For E) ★ ★ ★ P=0.0004. Abbreviations: ns, not significant; GFAP, glial fibrillary acidic protein; N-CAM, neural cell adhesion molecule; sph, spheres; adh, adherent; DMSO, dimethylsulfoxid.

★ ★

Figure 3. CD133 is upregulated in sphere cultures

Gene expression profiling (HuEx-1_0-st-v2) of two eRMS cells (RD and Rh36) and spheres (early, middle and late). A) left side: Cluster plot of RD and Rh36 cells and spheres. Right side: Analysis of RD samples. Genes, being up- or downregulated in RD spheres with a fold change of at least 2, are shown. B) Correlation plot of a metaanalysis performed with different publicly available expression data (hematopoietic and mesenchymal stem cells biopsies, FM95 cells, embryonic skeletal myoblast cells, embryonic stem cells, neuronal cells, glioblastoma spheres, cells and patient samples, neurospheres and prostate cancer samples) as indicated C) Expression of CD133 mRNA quantified by real-time PCR after correction with GAPDH levels as house-keeping gene. D) Flow cytometry analysis of CD133 (blue) expression. As controls unstained adherent and sphere cells were used, respectively (grey). E) Immunofluorescence staining of CD133 (green) of adherent and sphere cells. The nuclei were counterstained with DAPI (blue). Fields of two independent slides with at least 50 cells each were counted and the percentage of positive stained cells calculated. F) Western blot analysis of CD133 protein expression in adherent and sphere cells. ß-Tubulin was used as a loading control. A representative blot is shown. For C) ★ P=0.0284; ★ ★ P=0.0079. Abbreviations: ctrl, control; MSC, mesenchymal stem cells.

Figure 4. CD133$^+$ RMS cells are more chemoresistant and tumorigenic

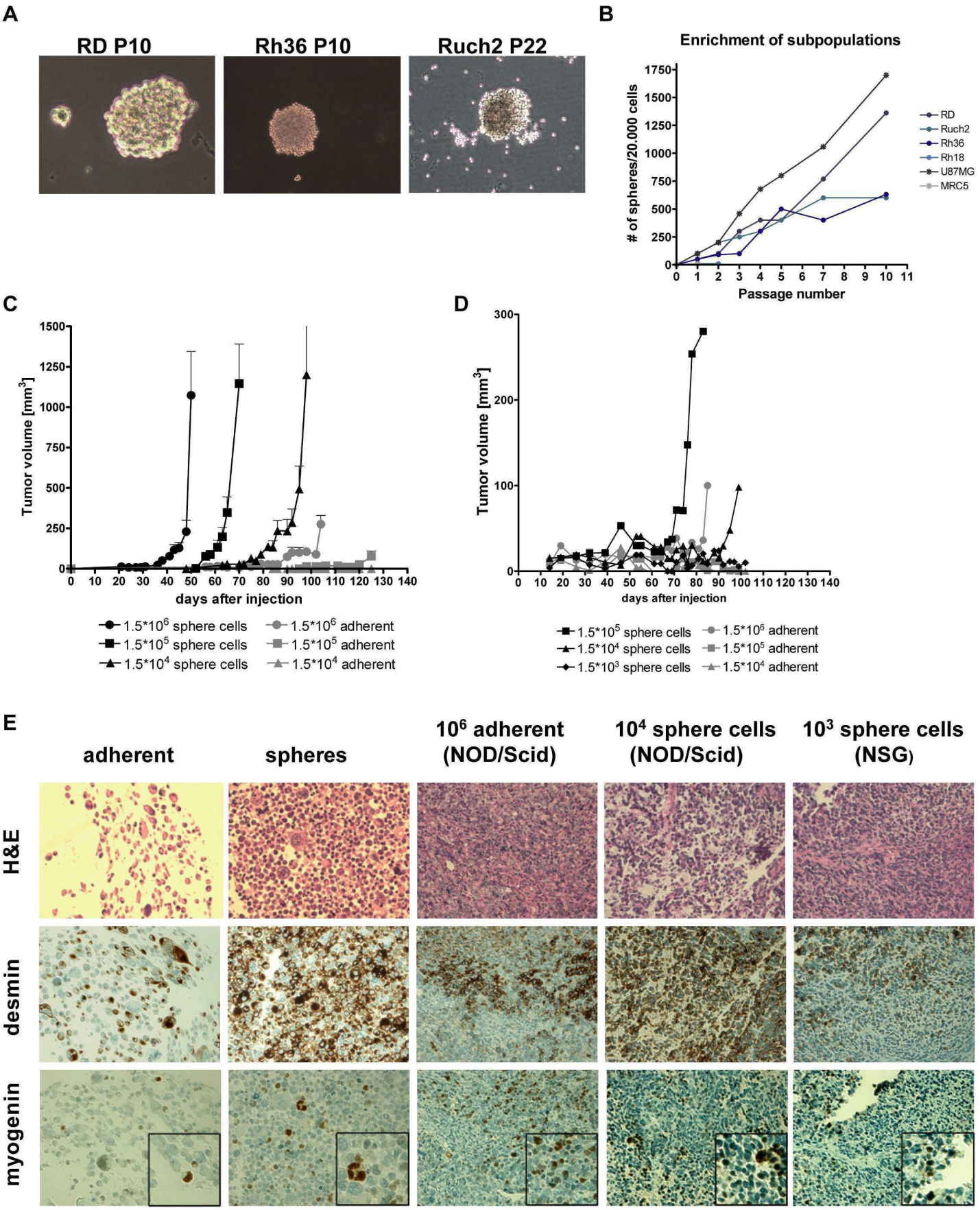
A) RD cells were stained for CD133 (blue) and sorted into CD133$^+$ (green) and CD133$^-$ (violet) populations with a MoFlo high speed cell sorter (DakoCytomation). Unstained RD cells were used as control (grey). After sorting the different fractions were reanalyzed by flow cytometry. B) Limited dilutions *in vivo* of different subpopulations (10^6, 10^5, 10^4, $10^3$and 10^2). Bulk stained (10^6) and unstained cells without sorting (10^6) were used as controls. Cells were injected i.m. into NOD/Scid mice (n=4) and tumor growth measured. Numbers indicate mice with growing tumors. C) Immunohistochemical (IHC) analysis of all xenograft tumors (H&E, Myogenin and Desmin). Representative stainings are shown. D) Clonogenic assay with sorted subpopulations (CD133$^+$ and CD133$^-$). Cells were treated with cisPlatin (IC10=10µM and IC60=50µM) and Chlorambucil (IC10=6.45µM). Colonies were visualized by crystal violet. cisPlatin: mean of 3 independent sortings ± SEM; Chlorambucil: mean of 2 independent sortings ± SEM. For D) ★ ★ P=0.0377; ★ P=0.0241. Abbreviations: IC, inhibitory concentration; ctrl, control.

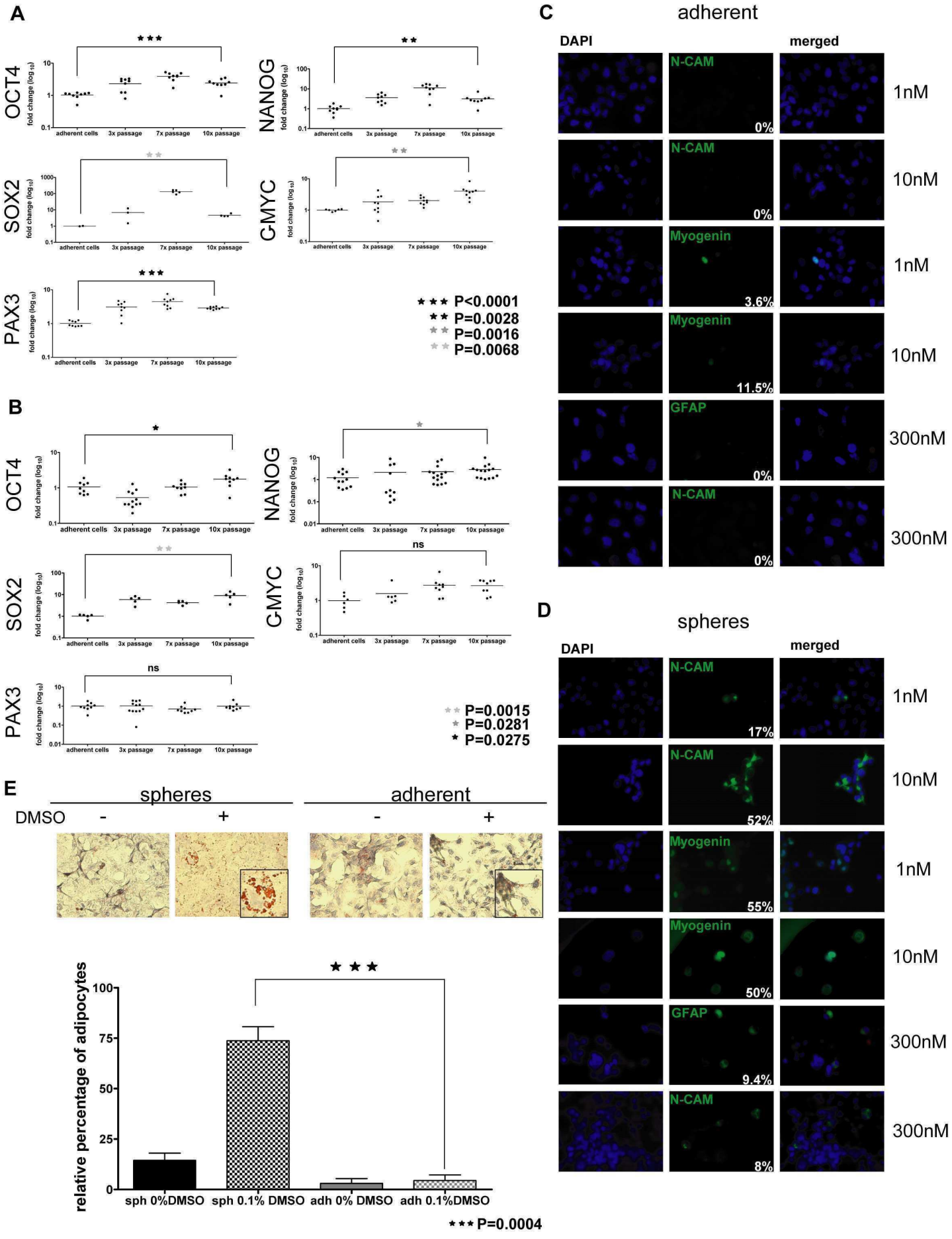
Figure 5. High expression of CD133 correlates with a poor survival rate

Immunofluorescence staining of a human RMS TMA with CD133 (green). Nuclei were counterstained with DAPI (blue). Two values were chosen for scoring: Staining intensity (1 = low, 2 = middle and 3 = bright) and number of positive cells (0=0; 1=1-10; 2=11-20; 3≥21). Both values were added up to the scorings negative (0 and 1), low (2 and 3), middle (4) and high (5 and 6). A) Stainings of representative tumor sections are shown for high, middle and low scorings. B) Overall survival of eRMS patients as shown by a Kaplan-Meier curve. For A) ★ ★ P=0.0272.

Beat W. Schäfer Figure 1

top

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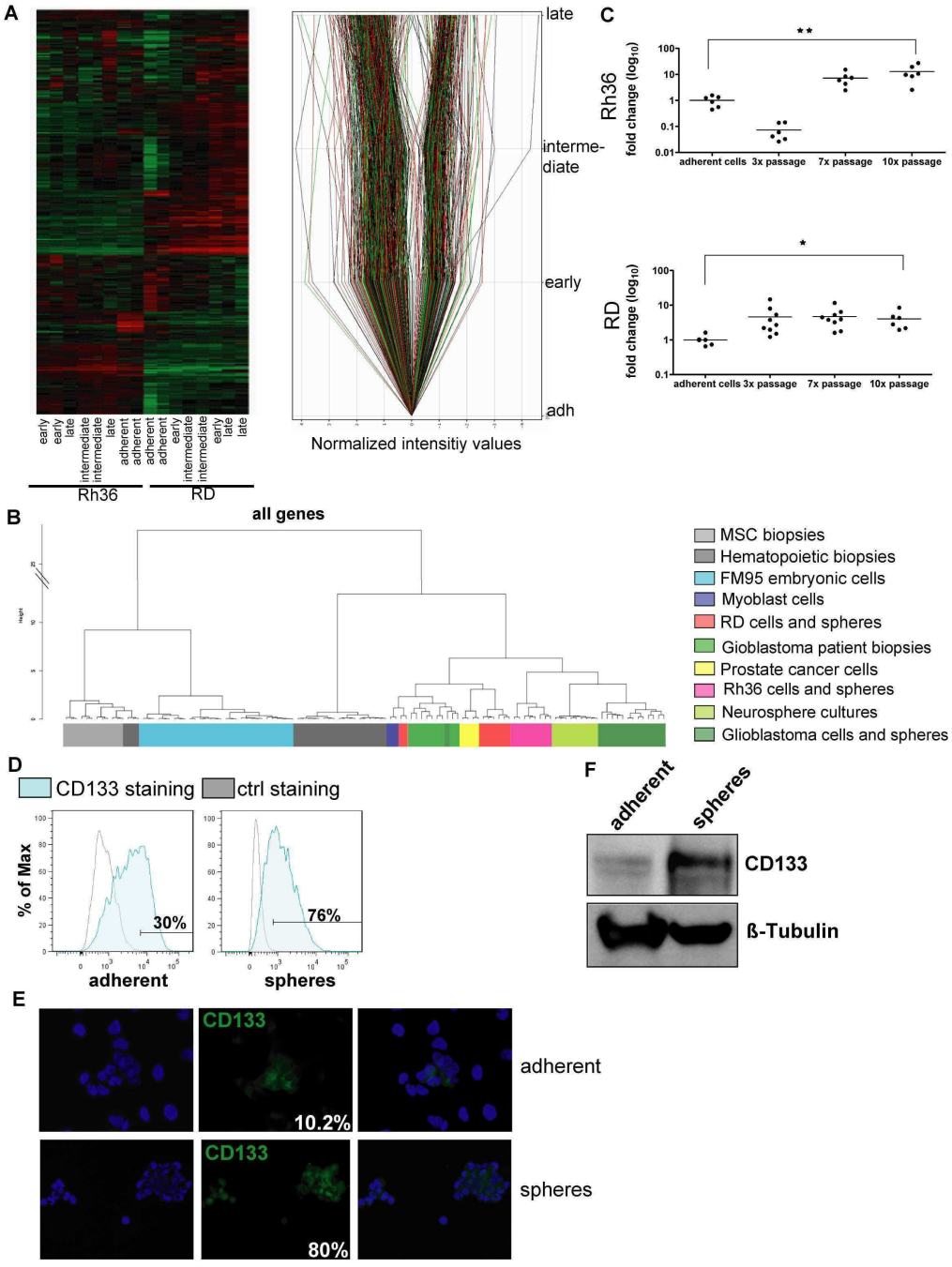
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10x passage
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all genes
MSC biopsies
Hematopoietic biopsies
FM95 embryonic cells
Myoblast cells
RD cells and spheres
Gioblastoma patient biopsies
Prostate cancer cells
Rh36 cells and spheres
Neurosphere cultures
Glioblastoma cells and spheres
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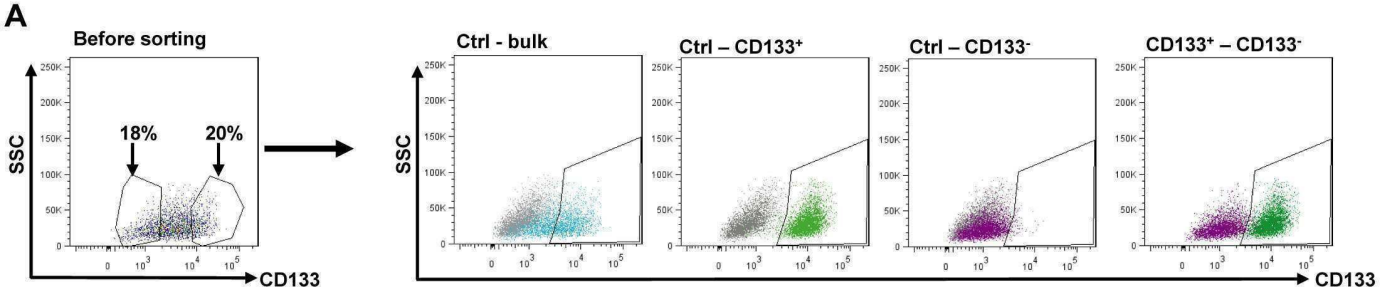
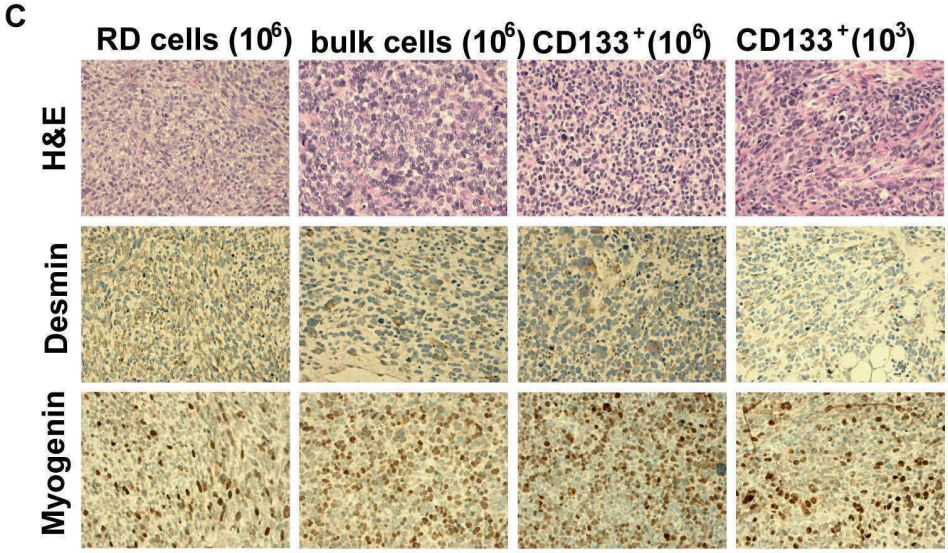


B

RD cells (10^6)	bulk cells (10^6)			
3 (4)	4 (4)			
CD133+ (10^6)	CD133+ (10^5)	CD133+ (10^4)	CD133+ (10^3)	CD133+ (10^2)
1 (1)	2 (4)	0 (4)	1 (4)	1 (4)
CD133- (10^6)	CD133- (10^5)	CD133- (10^4)	CD133- (10^3)	CD133- (10^2)
1 (1)	0 (4)	0 (4)	0 (4)	0 (4)

C



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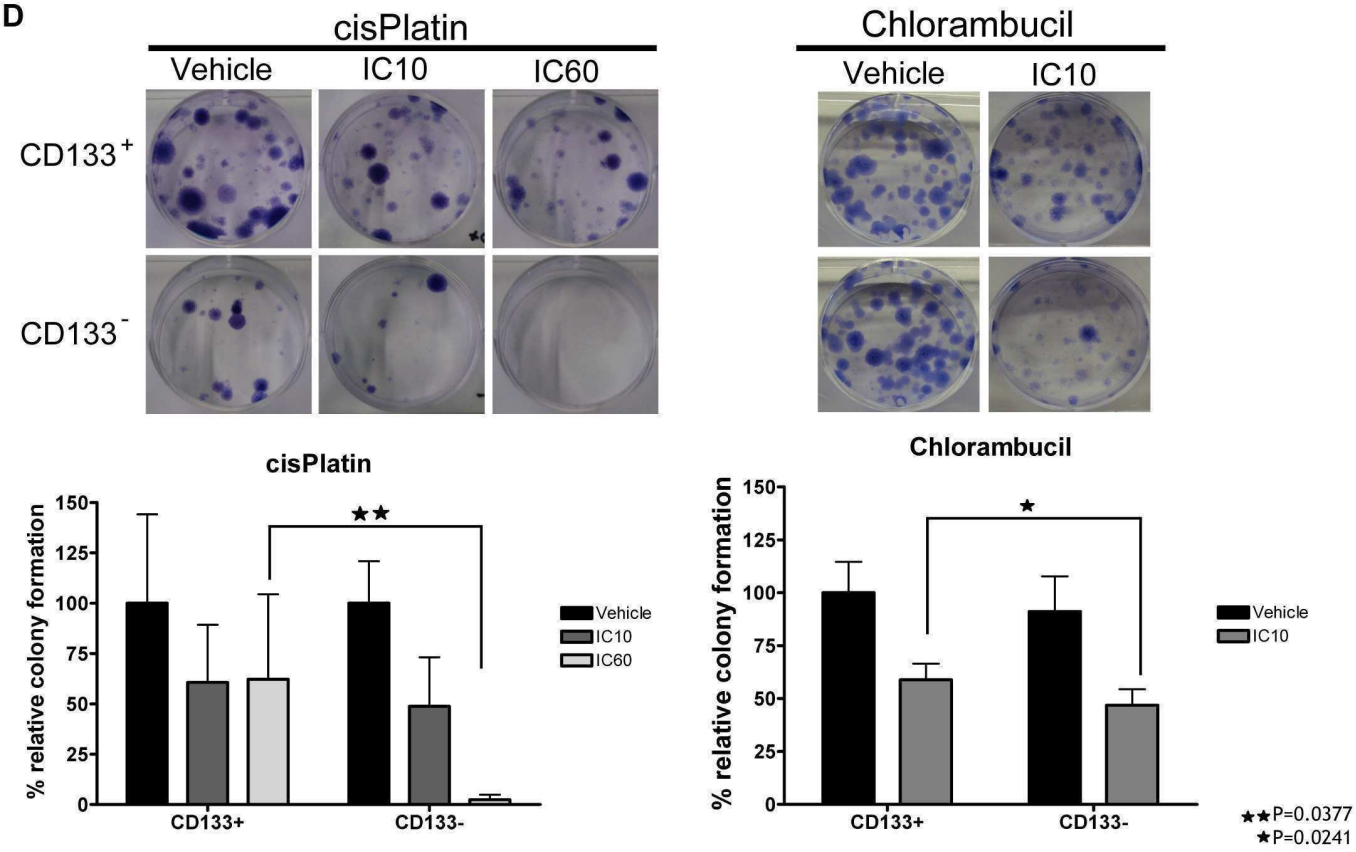
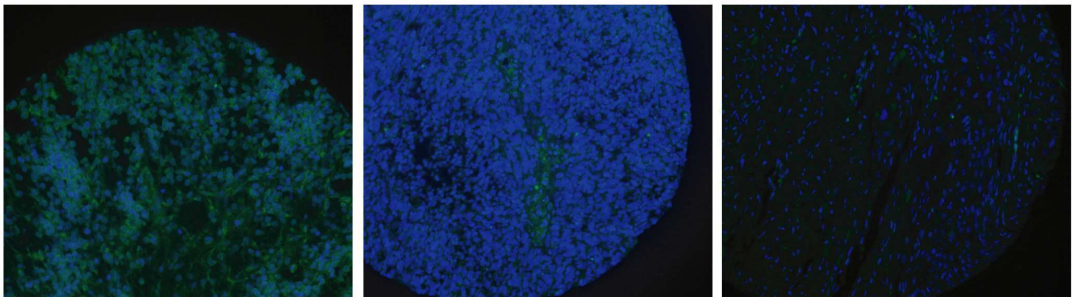


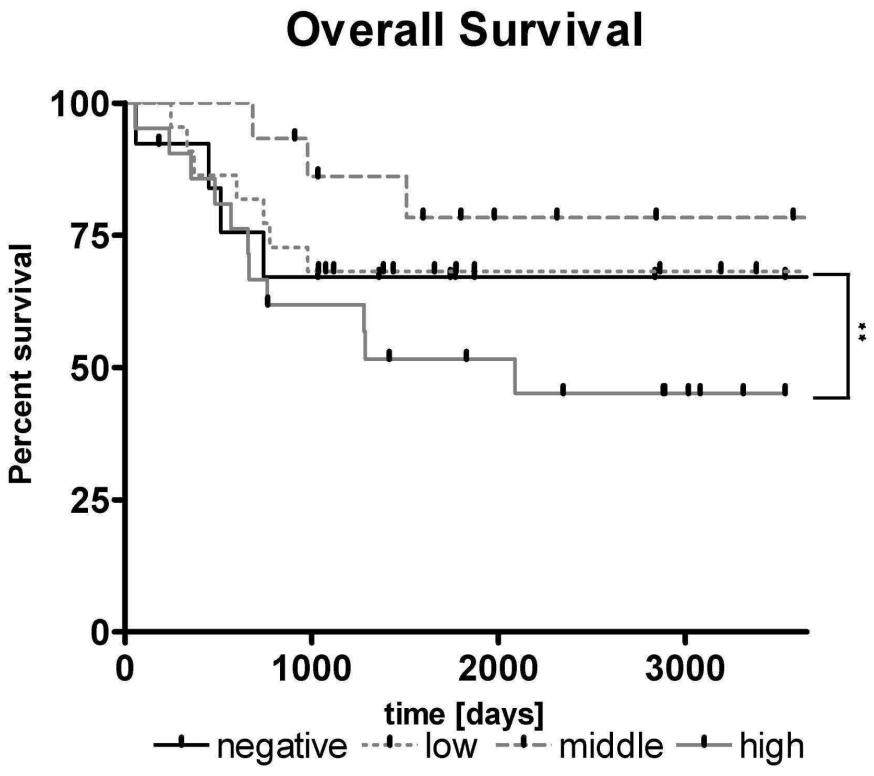
Beat W. Schäfer Figure 5 top

A



high middle low

B



** P=0.0272

6.2 Additional results

6.2.1 Cannabinoid receptor 1 is a potential drug target for treatment of translocation-positive rhabdomyosarcoma

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Dagmar Walter: Figure 1b, Figure 3a,b; Figure 6a and Discussion

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Kathya Prêtre: Figure 6a

Maria Salazar: Revision and Critical reading

Manuel Guzmán: Revision and Critical reading

Guillermo Velasco: Revision and Critical reading

Beat W. Schäfer: Principal Investigator; Conception; Discussion and Writing

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Cannabinoid receptor 1 is a potential drug target for treatment of translocation-positive rhabdomyosarcoma

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Abstract

Gene expression profiling has revealed that the gene coding for cannabinoid receptor 1 (CB1) is highly up-regulated in rhabdomyosarcoma biopsies bearing the typical chromosomal translocations PAX3/FKHR or PAX7/FKHR. Because cannabinoid receptor agonists are capable of reducing proliferation and inducing apoptosis in diverse cancer cells such as glioma, breast cancer, and melanoma, we evaluated whether CB1 is a potential drug target in rhabdomyosarcoma. Our study shows that treatment with the cannabinoid receptor agonists HU210 and Δ^9-tetrahydrocannabinol lowers the viability of translocation-positive rhabdomyosarcoma cells through the induction of apoptosis. This effect relies on inhibition of AKT signaling and induction of the stress-associated transcription factor p8 because small interfering RNA–mediated down-regulation of p8 rescued cell viability upon cannabinoid treatment. Finally, treatment of xenografts with HU210 led to a significant suppression of tumor growth *in vivo*. These results support the notion that cannabinoid receptor agonists could represent a novel targeted approach for treatment of translocation-positive rhabdomyosarcoma. [Mol Cancer Ther 2009;8(7):1838–45]

Introduction

Rhabdomyosarcoma (RMS) is the most common soft-tissue sarcoma in children, representing 5% to 8% of all childhood malignancies (1). It is believed to originate from muscle precursor cells and histology recognizes two major subtypes: The embryonal subtype (eRMS) accounts for ~60% of RMS cases and has a rather good prognosis (2). The alveolar subtype (2) is less frequent, more aggressive, usually presents with metastasis, and is thus associated with rather poor treatment outcome. Although no consistent genetic alterations have been identified thus far in eRMS, ~80% of aRMS patients display typical chromosomal translocations t(2;13)(q35;q14) or t(1;13)(p36;q14) encoding for fusion proteins PAX3/FKHR or PAX7/FKHR, respectively (3). These chimeric transcription factors are oncogenic and presumably act mainly through their gain in transcriptional activity. To provide insight into molecular changes elicited by these transcription factors and to find new potential therapeutic targets for treatment of aRMS, gene expression analysis was done in a range of RMS biopsies by several research groups (4–6). These studies consistently revealed a gene expression signature of up-regulated genes in translocation-positive versus translocation-negative samples. Interestingly, translocation-negative aRMS clustered together with eRMS samples in these analyses. Hence, at the molecular level, RMS can be divided into translocation-positive RMS (tposRMS) and translocation-negative RMS (tnegRMS).

The gene expression signature of tposRMS contains a number of receptor molecules that might be potentially amenable as drug targets. Among these, receptors such as c-met have already been validated as therapeutic target (7). However, one of the top-ranking genes in this signature is the cannabinoid receptor 1 (CB1). Thus far, no studies have been undertaken to assess whether CB1 might serve as a future target for therapeutic intervention in this tumor. Evidence exists since 1975 that cancer cell growth can be inhibited by treatment with cannabinoid receptor agonists, as first described by Munson et al. in Lewis lung carcinoma cells (8). Since then, additional cancer cell types such as glioblastoma (9), breast carcinoma (10), or melanoma (11) were reported to be sensitive to the antiproliferative action of cannabinoids. In general, the antitumoral actions of diverse cannabinoid receptor agonists are mediated through the cannabinoid receptor types CB1 and CB2, as reviewed by Guzman et al. (12). Notably, not only *in vitro* cell culture systems are subject to this treatment response but also *in vivo* experiments using either xenografts or syngeneic mouse models have shown the potential of cannabinoids as anticancer agents, without observing major psychoactive or immune-suppressive effects (8, 13). Recently, the first clinical study using Δ^9-tetrahydrocannabinol (THC) in severe cases of glioblastoma has been reported (14).

At the molecular level, cannabinoids trigger changes in various signaling pathways in cancer cells. One of the primary events after cannabinoid treatment is a sustained *de novo* synthesis of the lipid second messenger ceramide, which in turn is followed by inhibition of AKT signaling (15). Strikingly, both of these signaling events mark a

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major difference between tumor cells and healthy nontransformed cells, which undergo AKT activation without *de novo* synthesis of ceramide after cannabinoid stimulation (16). In parallel to AKT inhibition, alterations in extracellular signal-regulated kinase (ERK) signaling have been reported. However, depending on tumor type, either inhibition (17) or sustained activation (18) has been observed. Recently, the stress-associated transcription factor p8 was found to be critically involved in cannabinoid-induced apoptosis of cancer cells (19) as its down-regulation could rescue viability of various cancer cells (20, 21). At the end of the signaling cascade, tumor cells either undergo cell cycle arrest (22, 23) or apoptosis (9, 24).

To improve the treatment outcome of the aggressive tposRMS subtype, novel targeted therapies are urgently needed. Therefore, this study aimed to characterize the effects of cannabinoids on tposRMS cells *in vitro* as well as *in vivo*. Our results show that the CB1 receptor could represent a potential molecular target for future therapeutic approaches in tposRMS.

Materials and Methods

Cannabinoids

HU210 [(−)-1,1-dimethylheptyl analogue of 11-hydroxy-Δ^8-tetrahydrocannabinol] was purchased from Tocris, 2-methyl-2′-*F*-anandamide (Met-F-AEA) from Cayman, AM251 (analogue of SR141716A) from Sigma-Aldrich, and THC from The Health Concept. All substances were solved in DMSO. For *in vitro* experiments, they were applied at final DMSO concentrations of maximally 0.05% (v/v). For *in vivo* experiments, HU210 was prepared at 0.25% DMSO (v/v) and diluted in PBS supplemented with 5 mg/mL bovine serum albumin.

Cell Culture

Rh4 and Rh28 tposRMS cells were kindly provided by P. Houghton (St Jude Children's Research Hospital, Memphis, TN, USA). RMS13, RD, and MRC-5 lung fibroblast cells were obtained from the American Type Culture Collection (LGC Promochem). All cells were routinely maintained in DMEM supplemented with 10% FCS. When performing viability or signaling experiments, cells were plated at a density of 17,000/cm^2, allowed to adhere overnight, and transferred to serum-free medium 6 h before starting drug treatments.

Cell Viability and Apoptosis Detection

Cell viability was evaluated in 96-well plates using methylthiazolyldiphenyl-tetrazolium bromide from Sigma-Aldrich. Apoptosis was either analyzed with CaspGLOW red active caspase-3 staining kit (Biovision), allowing labeling of apoptotic cells with a fluorescent caspase-3 substrate and subsequent detection by fluorescence microscopy. Alternatively, the apoptosis-indicating ratio of cleaved to uncleaved poly(ADP-ribose)polymerase (PARP) protein was determined densitometrically by Western blotting.

Reverse Transcriptase-PCR

RNA was extracted with the RNeasy Mini Kit (Qiagen), including a DNase digestion step with RNase-free DNase (Qiagen). One microgram of total RNA was reverse transcribed with random hexamer primers using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). PCR was done with primers for hCB1 (5′-CGTGGGCAGCCTGTTCCTCA-3′ and 5′-CATGCGGGCTTGGTCTGG-3′) and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Microsynth) using the following parameters: After initial denaturation at 94°C for 5 min, cycles (40×) with 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s was done, followed by a final extension at 72°C for 5 min.

Quantitative Real-time PCR

Quantitative reverse transcription-PCR (RT-PCR) was done under universal cycling parameters on an ABI7900HT instrument using commercially available Mastermix and target probes for CB1, p8, and GAPDH (all from Applied Biosystems). Cycle threshold (C_T) values were normalized to GAPDH. Relative expression levels of the target genes among the different samples were calculated using the $\Delta\Delta C_T$ method.

Gene Silencing

Rh4 cells were transfected with 10 nmol/L small interfering RNA (siRNA) against human p8 (Qiagen) or scrambled siRNA (Ambion) using GeneEraser (Stratagene). One day after siRNA transfection, equal numbers of cells were plated for subsequent viability experiments. p8 down-regulation efficiency was verified by means of quantitative RT-PCR.

Western Blot Analysis

For detection of intracellular signaling proteins, whole-cell extract was produced with a lysis buffer consisting of 50 mmol/L Tris (pH 7.5), 1% Triton X-100, 1 mmol/L EGTA, 50 mmol/L NaF, 10 mmol/L sodium β-glycerophosphate, 5 mmol/L sodium PPi, 1 mmol/L sodium orthovanadate, 1% phenylmethylsulfonyl fluoride, 0.1% β-mercaptoethanol, and protease inhibitor cocktail complete (Roche) according to standard protocols. Samples were sonicated and equal amounts of protein were used for Western blotting with the NuPAGE system (Invitrogen). Antibodies used for detection included rabbit antibodies raised against CB1 (1:1,000; Affinity Bioreagents), PARP, phospho-AKT (Ser^{473}), phospho-AKT (Thr^{308}), AKT-total, phospho-ERK (Thr^{202}/Tyr^{204}), ERK-total, phospho-GSK (Ser$^{21/9}$), and GSK (all 1:1,000; from Cell Signaling Technology). Detection of actin with a rabbit antibody (1:2,000; Sigma-Aldrich) was used to control for equal protein loading. As secondary antibody, an anti-rabbit antibody conjugated to horseradish peroxidase (1:2,000; Pierce) was used. Detection was done with ECL technology (Amersham).

Confocal Microscopy

Cells on coverslips were fixed with paraformaldehyde (PFA) and incubated with anti-CB1 antibody (1:500; Affinity Bioreagents) in PBS/2.5% goat serum for 0.5 hours at 37°C. For visualization, a secondary anti-rabbit antibody labeled with Alexa Fluor 594 (1:500; Molecular Probes) was used. Control immunostainings using the secondary antibody alone were done in parallel. Confocal fluorescence images were acquired using Laser Sharp 2000 software (Bio-Rad) and a Confocal Radiance 2000 coupled to an Axiovert S100 TV microscope (Carl Zeiss).

Immunohistochemical Staining

Tumors were fixed, embedded in paraffin, and sectioned into 2-μm slices. Immunohistochemical stainings for Ki67 (Lab Vision Corporation) and cleaved caspase-3 (Cell Signaling Technology) were done on the Ventana Benchmark automated staining system (Ventana Medical Systems).

Tumorigenicity Assay

Rh4 cells (7.5×10^6) in 100 μL PBS were injected s.c. into the flank of NOD/LtSz-scid IL2Rγ null (NOG) mice (The Jackson Laboratory). When tumors reached a size of 150 mm^3, mice were randomly assigned to treatment and control groups and injected peritumorally for 13 d with 0.2 mg/kg HU210 or vehicle (DMSO) alone. Tumor growth was monitored daily with external caliper, and the tumor volume was calculated as $(4\pi/3)((\text{width} + \text{length})/4)^3$. Animals were sacrificed 1 d after the last treatment.

Statistical Analysis

Statistical analysis was done with two-tailed *t* test with the statistics program SPSS. For analysis of tumor growth, a longitudinal analysis was done by comparing linear regressions of the two groups.

Results

The CB1 Receptor Is Up-Regulated in Translocation-Positive RMS

Gene expression profiling of RMS biopsy samples shows a signature for tposRMS (4) that includes, as one of the top-ranking genes, *CNR1*, encoding the CB1 receptor (Fig. 1A). In contrast, transcript levels of the related *CNR2* gene, encoding the CB2 receptor, were only slightly above background. To validate the up-regulation of CB1 in tposRMS cells on both the RNA and protein levels, we first applied conventional RT-PCR, which revealed a higher expression of CB1 mRNA in all tposRMS cells than in control cell lines MRC-5 (fibroblast) and RD (eRMS; Fig. 1B). Indeed, expression was >10,000-fold higher than in the controls when assessed quantitatively. In addition, expression of CB1 is 430-fold higher than expression of CB2 inRh4 cells, whereas in U87MG glioma and A375 melanoma cells, expression of CB2 is more prevalent (data not shown). Further, CB1 was expressed in Rh4 cells also at the protein level as shown by Western blot (Fig. 1C). Last, confocal microscopy of cultured Rh4 and RD cells (Fig. 1D) showed higher immunofluorescence staining intensities for CB1 in Rh4 cells. Hence, expression of CB1 is evident both on the mRNA and protein levels in tposRMS cells, confirming the previous findings using gene expression profiling.

Cannabinoids Reduce the Viability of tposRMS Cells *In vitro*

After validating the expression of CB1 in tposRMS cells, we next assessed the cell viability after treatment with different cannabinoid receptor agonists. Treatment with the mixed cannabinoid receptor agonist HU-210 reduced the viability of two tposRMS cell lines in a dose-dependent manner (Fig. 2A). Similarly, the main active component of marijuana (THC) as well as the anandamide-related compound Met-F-AEA reduced the viability of Rh4 cells in a dose-dependent manner but not of the tnegRMS cells (RD) or control nontransformed fibroblasts (MRC-5), which express lower levels of the CB1 receptor (Fig. 2C and D). Finally, pharmacologic blockade of the CB1 receptor significantly restored cell viability of Rh4 cells from 29.2% (±2.4SD) to 71.9% (±11.2 SD; Fig. 2B), supporting the notion that the observed reduction in cell viability was specifically mediated through the CB1 receptor.

Cannabinoids Induce Apoptosis in tposRMS Cells

To determine whether decreased cell viability in tposRMS cells after cannabinoid treatment is due to apoptosis, caspase-3 activation and PARP cleavage were analyzed. First, tposRMS cells were treated with 1.25 μmol/L HU210 and cell extracts

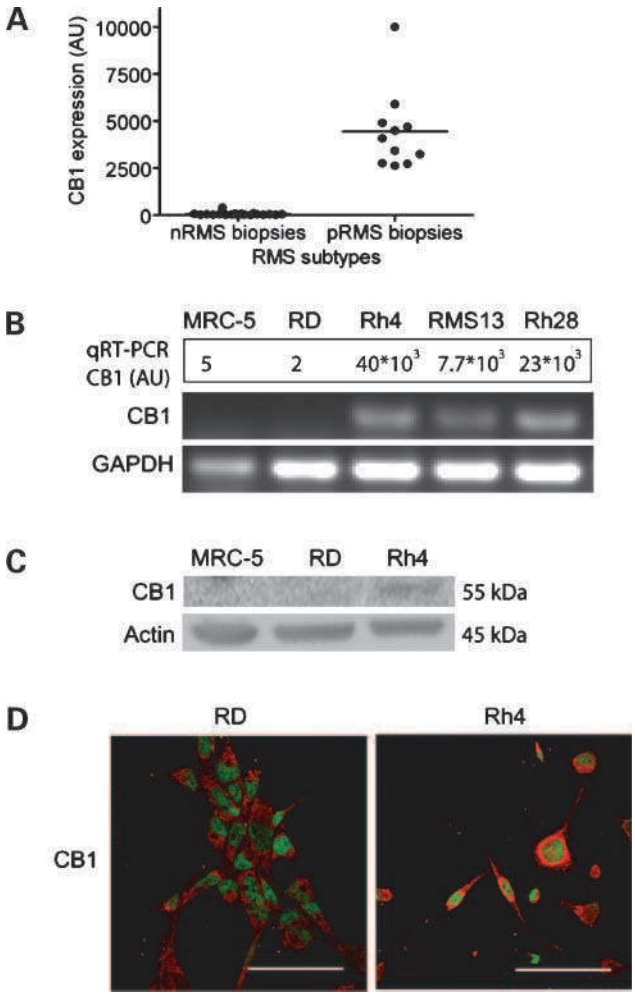

Figure 1. CB1 expression in tposRMS cells. **A,** gene expression values of CB1 are shown in arbitrary units. Samples analyzed by microarray gene expression profiling were translocation-negative (*tnegRMS*) versus translocation-positive (*tposRMS*) biopsy samples. **B,** quantitative and normal RT-PCR with primers for CB1 and for GAPDH were done with cDNA of cell lines MRC-5 (fibroblast); RD (tnegRMS); and Rh4, Rh28, and RMS13 (all tposRMS) cells. Quantitative results are indicated in arbitrary units. **C,** CB1 protein levels of MRC-5, RD, and Rh4 cells were determined by Western blotting. **D,** confocal images of immunofluorescence stainings with anti-CB1 antibody (*red fluorescence*) on RD and Rh4 cells (*scale bar,* 100 μm).

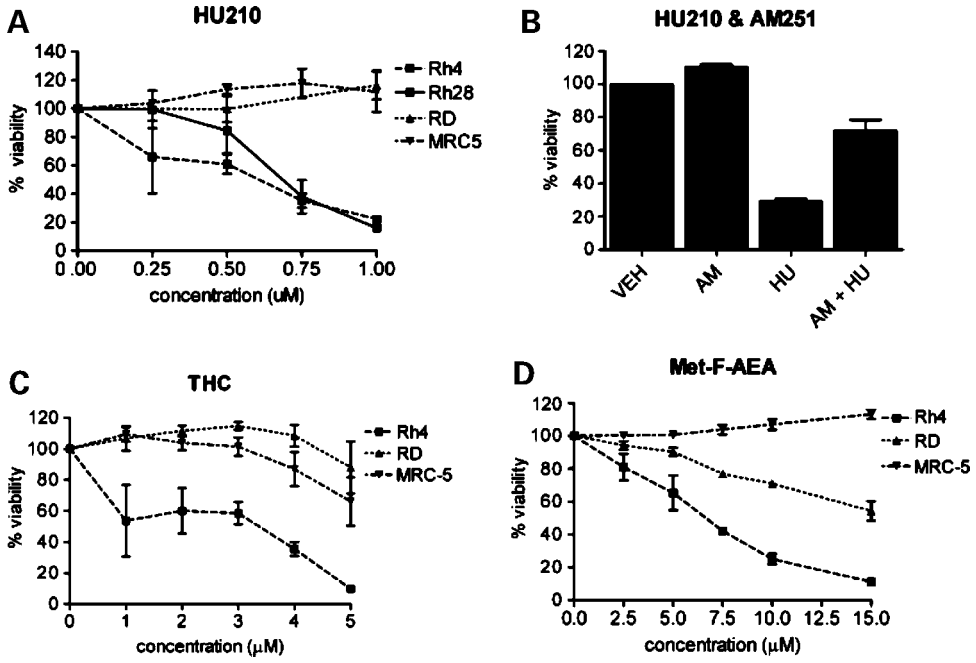

Figure 2. Cannabinoids reduce viability of tposRMS cells. **A,** cell lines Rh4, Rh28 (tposRMS), RD (tnegRMS), and MRC-5 (fibroblasts) were incubated with increasing concentrations of HU210 for 72 h. Subsequent viability measurements by means of MTT are shown (n = 3, ±SE; significance at 1.25 μmol/L: $P < 0.005$). **B,** viability measurements are shown for Rh4 cells preincubated with vehicle or with 0.5 μmol/L AM251 before undergoing subsequent treatment with 1 μmol/L HU210 for 24 h (n = 3, ±SE, significance: $P < 0.05$). **C** and **D,** dose-dependent viability of Rh4, RD, and MRC-5 cells after treatment with THC for 24 h or Met-F-AEA for 48 h was measured (n = 3, ±SE, significance at 5 μmol/L THC and 10 μmol/L Met-F-AEA: $P < 0.05$).

were analyzed by immunoblotting for PARP cleavage at different time points (Fig. 3A). Already 6 hours after start of treatment, PARP cleavage could be observed and after 24 hours, almost no uncleaved protein was detectable. Treatment of Rh4 and Rh28 cells with increasing HU210 concentrations showed a similar increase in PARP cleavage, whereas pretreatment of Rh4 cells with 0.5 μmol/L of the CB1-specific antagonist AM251 significantly rescued cleavage of PARP protein (Fig. 3B). At a concentration of 1.25 μmol/L HU210, for example, the ratio of cleaved to uncleaved PARP protein could be rescued from 1.13 (±0.05 SD) to 0.40 (±0.03 SD; Fig. 3C).

Additionally, caspase-3 activation after 24 hours of HU210 treatment was measured in Rh4 cells (Fig. 3D). A concentration-dependent increase of cells positively stained for active caspase-3 was detected with close to 100% apoptotic cells at 1.25 μmol/L of HU210. In line with the previous results, we also observed that both THC (2 and 4 μmol/L) and Met-F-AEA (5 and 10 μmol/L) treatment induced cleavage of PARP protein after 24 hours of incubation (Fig. 3E and F). Therefore, treatment of tposRMS with cannabinoids induces apoptosis in tposRMS cells.

Cannabinoids Inhibit AKT Signaling

Earlier studies investigating effects of cannabinoids on cancer cells could show alterations in AKT and ERK signaling upon drug treatment. Based on this, we next studied AKT and ERK signaling in tposRMS cells after cannabinoid treatment. Among the tposRMS cell lines, Rh4 cells most accurately reflect the translocation-specific gene expression signature and therefore this cell line was selected as model system for further studies. They were incubated with 1.25 μmol/L HU210 for 30 minutes and 2 hours before cell lysis. A rapid decrease in phospho-AKT at Ser^{473} was detected, indicating inhibition of AKT activity (Fig. 4A). Under the same experimental conditions, phosphorylation of ERK was found to increase in tposRMS cells after drug treatment compared with vehicle-treated cells, at both Thr^{202}/Tyr^{204} (Fig. 4A, *bottom left*). Further phosphorylation on Thr^{308} of AKT was also reduced. In addition, the AKT downstream target GSK3β became significantly dephosphorylated (Fig. 4A, *right*). Notably, also THC (2 and 4 μmol/L) as well as Met-F-AEA (5 and 10 μmol/L) triggered dephosphorylation of AKT at Ser^{473} (Fig. 4B) with a slight delay compared with HU210. No difference for ERK phosphorylation was observed with either of these two substances (data not shown). In summary, all three cannabinoid agonists lead to inhibition of AKT signaling in tposRMS cells, whereas ERK activation was only seen after treatment with HU210. These experiments suggest that the AKT pathway is likely to mediate the action of cannabinoids in our tumor model.

Cannabinoids Reduce Viability through Up-Regulation of Transcription Factor p8

p8 is a transcription factor involved in cellular stress responses following cellular injuries through pathways implicated in growth inhibition (25, 26). Furthermore, p8 mediates apoptosis upon cannabinoid treatment of glioblastoma (19), pancreatic cancer (20), and breast cancer (21) cells. Therefore, we tested involvement of p8 in the antiproliferative action of HU210, THC, and Met-F-AEA in our model. p8 levels were assessed in mRNA isolated 16 hours after the addition of drugs to Rh4 cells. A clear dose-dependent increase in p8 transcripts up to 6.5-fold was observed for all cannabinoids used compared with vehicle-treated control samples (Fig. 5A).

To validate the requirement of p8 up-regulation for induction of apoptosis, p8 expression was specifically down-regulated by treatment with siRNA. mRNA levels after treatment were on average down to 16% (±5.5% SD) 48 hours after transfection. Upon incubation with 1.25 μmol/L of HU210 for 48 hours, viability of scrambled-transfected cells was reduced to 37% (±9.4 SE), whereas cells with lower p8 transcript levels showed a rescue in viability up to 63% (±8.5 SE; Fig. 5B). This suggests that an increase in p8 levels after cannabinoid treatment is an important component of the molecular response. These experiments indicate that p8 mediates, at least in part, the reduction in viability observed after cannabinoid treatment.

HU210 Reduces Tumor Growth of tposRMS Xenografts

To test whether HU210 might have a therapeutic effect on tposRMS tumors *in vivo*, tumor xenografts were generated by s.c. injection of Rh4 cells into immunodeficient NOG mice. Tumors were treated peritumorally with HU210 daily for 13 subsequent days. We observed significantly reduced tumor growth in HU210-treated compared with vehicle-treated animals (Fig. 6). Tumors were excised after the last day of treatment and paraffin-embedded sections were

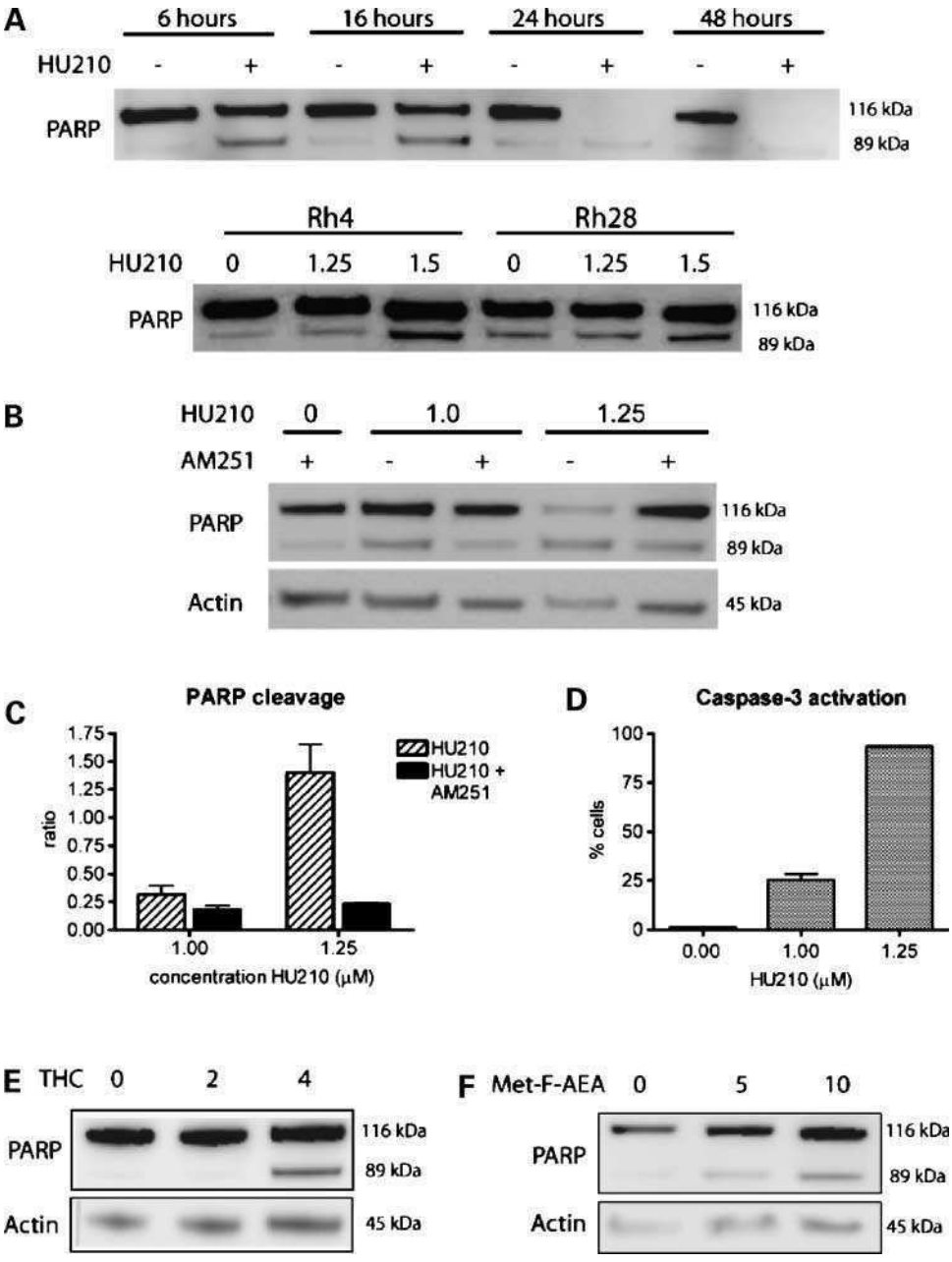

Figure 3. Cannabinoids induce apoptosis in tposRMS cells. **A,** Rh4 cells were treated for 6, 16, 24, and 48 h with either 1.25 μmol/L HU210 or DMSO. Rh28 and Rh4 cell were incubated with increasing concentrations of HU210 for 24 h (*bottom*). Subsequently, Western blotting was done with an anti-PARP antibody. **B,** after preincubation of Rh4 cells with 0.5 μmol/L of CB1 antagonist AM251, HU210 was added at concentrations of 1 and 1.25 μmol/L HU210 for 20 h. Cell lysates were probed with anti-PARP (*top*) and anti-actin (*bottom*) by immunoblotting. **C,** densitometric quantification of the ratio of cleaved to uncleaved PARP product (values ± SE, $n = 2$). **D,** percentage of cells staining positively for proapoptotic caspase-3 is shown as evaluated after 20 h of HU210 treatment of Rh4 cells (values ± SE, $n = 3$, significance $P < 0.005$). Rh4 cells were either treated with 2 and 4 μmol/L of THC (**E**) or 5 and 10 μmol/L of Met-F-AEA (**F**) for 24 h. Protein extract was analyzed for PARP and actin by Western blotting (here shown a representative blot).

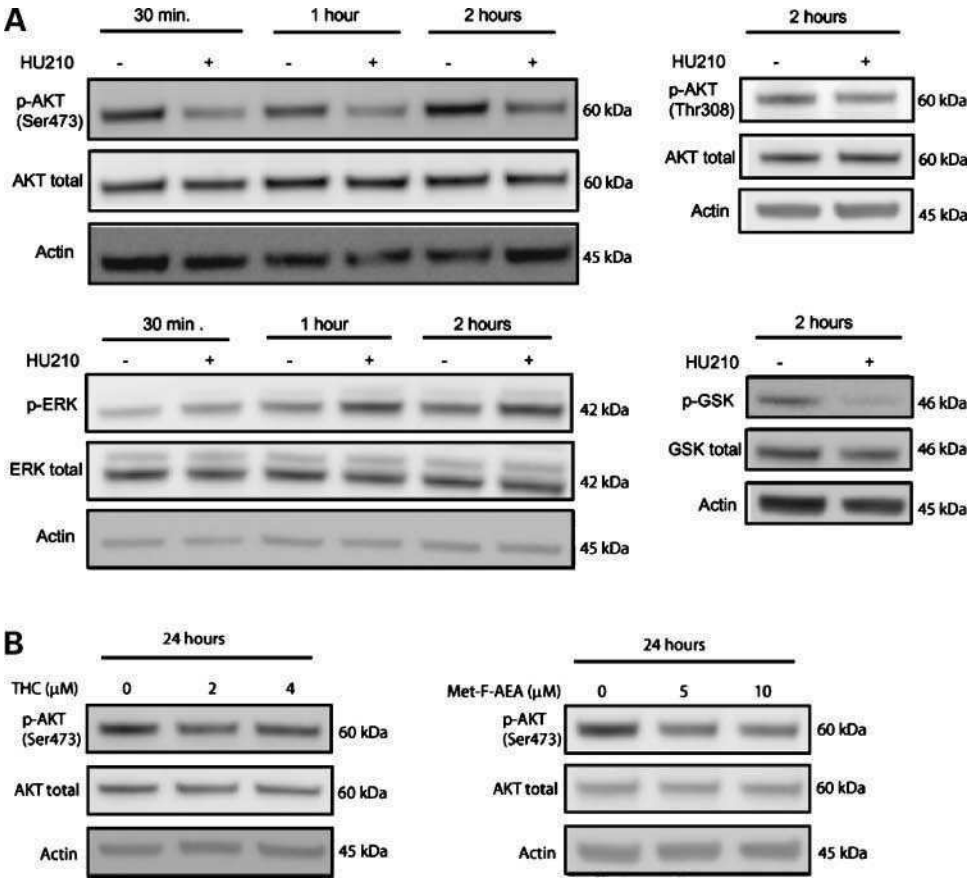

Figure 4. Cannabinoid receptor agonists affect AKT and ERK signaling in tposRMS cells. **A,** Rh4 cells were incubated with 1.25 μmol/L HU210 for 30 min, 1 h, and 2 h, and cell lysates were prepared. Then, Western blotting was done with antibodies against phospho-AKT (Ser^{473}; *top left*) and against phospho-ERK (*bottom left*). Anti–phospho-GSK (*bottom right*) and anti–phospho-AKT (Thr^{308}; *top right*) were probed on extracts of cells treated for 2 h with 1.25 μmol/L of HU210. **B,** phosphorylation status of AKT at Ser^{473} was analyzed by Western blotting after treatment of Rh4 cells with 2 and 4 μmol/L THC (*top*) or 5 and 10 μmol/L Met-F-AEA (*bottom*) for 24 h.

either H&E stained or subsequently immunohistochemically analyzed with antibodies against the proliferation marker Ki67 and the apoptosis indicator cleaved caspase-3. H&E-stained sections from HU210-treated animals displayed a high number of cell-free patches filled with connective tissue, which are probably remains of previously apoptotic or necrotic areas. In agreement with this, a moderate increase of apoptotic cells, which was variable across tumors, was detected in HU210-treated mice compared with vehicle-treated animals (data not shown). On the other hand, no difference in the staining pattern for Ki67 was observed among treatment modalities (data not shown). In conclusion, HU210 is capable of reducing aRMS xenograft growth through induction of apoptosis *in vivo*.

Discussion

Evidence from *in vitro* and *in vivo* experiments suggests that cannabinoid receptor agonists can reduce tumor growth and induce apoptosis in several tumor types, including melanoma, breast and prostate cancer, colon cancer, leukemia, and glioma. However, to our knowledge, the response to cannabinoid treatment has not been studied in sarcomas yet. Here, we investigated the effects of cannabinoid receptor agonists in the sarcoma tposRMS, which we not only confirmed to express high levels of CB1 mRNA but also showed expression on the protein level by Western blot and immunohistochemistry.

In vitro, cannabinoid receptor agonists HU210, THC, and Met-F-AEA exerted an antiproliferative and proapoptotic action on tposRMS cells through activation of the CB1 receptor. The specificity of this effect for CB1 was shown by two means: First, the cell viability in fibroblasts or tnegRMS control cell lines, which express only low levels of CB1, is not affected. Second, the CB1-specific antagonist AM251 was able to significantly reduce apoptosis and partially restore cell viability. TposRMS cells were most sensitive to submicromolar concentrations of HU210, THC, and Met-F-AEA, and comparable with those observed in other cancer cells such as pancreatic cancer (20), breast cancer (22), or colon cancer (27) cells.

Key events contributing to cannabinoid-triggered induction of apoptosis in tposRMS cells are diminished AKT signaling and up-regulation of the transcription factor p8. Whereas cancer cells such as melanoma (11), colon cancer (17), and glioma (15) also experience dephosphorylation of AKT after cannabinoid stimulation, nontransformed CB1-expressing cells such as neurons react with increased phosphorylation of AKT under the same circumstances (16, 28). The key event responsible for this fundamental difference is still unknown; however, *de novo* ceramide synthesis (29) seems to be important for induction of apoptosis in cancer

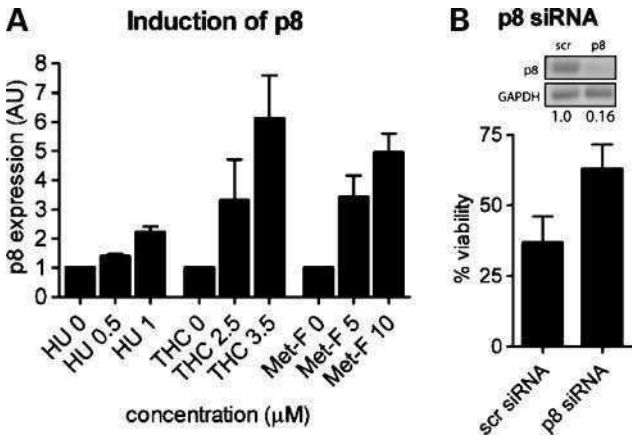

Figure 5. Induction of proapoptotic p8. **A,** Rh4 cells were treated with 0.5 and 1 μmol/L of HU210, 2.5 and 3.5 μmol/L of THC (**B**), or 5 and 10 μmol/L of Met-F-AEA for 16 h. RNA was extracted and analyzed for p8 transcripts with quantitative RT-PCR ($n = 3$, ±SE, $P < 0.05$). Values were normalized to GAPDH. **B,** p8 was down-regulated by means of siRNA. *Top,* a representative RT-PCR and quantitative values (in arbitrary units, normalized to scrambled siRNA transfected control cells). Viability after HU210 (1.25 μmol/L) treatment was assessed at 48 h with MTT ($n = 3$, ±SE, $P < 0.05$).

cells. Apart from AKT, the transcription factor p8 was recently shown to be up-regulated by cannabinoid receptor agonists and this event seems to be crucial for their sensitivity (19) because knockdown of this gene could rescue cell viability in cancer cells such as glioma (19), breast cancer (21), or pancreatic cancer (20). As shown here, also in tposRMS cells is p8 a critical mediator of proapoptotic signaling after cannabinoid treatment because inhibition of its accumulation by means of RNA interference significantly rescued cell viability. In contrast, the response of the ERK pathway is not consistent and seems to be either tumor type specific depending on the type of agonist used. Therefore, it is less likely to play an important role in our model.

Thus far, HU210 has been used in animal models to investigate neurogenesis (30) and multiple sclerosis (31) and was recently shown to prevent formation of preneoplastic lesions in mouse colon (32). However, HU210 treatment of xenograft-bearing mice has not been reported thus far. Here, we observed significantly reduced tumor growth in HU210-treated animals without overt psychoactive signs. Growth reduction observed was comparable with other xenograft models treated with cannabinoids, such as treatment of pancreatic cancer tumors with THC or JWH-133 (20). A moderate increase in the number of apoptotic cells was observed in HU210-treated xenograft sections; however, we cannot exclude other mechanisms to additionally account for reduction in tumor growth. However, analysis of transcript levels of myogenic differentiation markers, such as myosin light chain or troponin C (33), did not significantly differ between treatment modalities, ruling out the possibility that cannabinoids induce differentiation in tposRMS cells as observed after inhibition of PAX3/FKHR function.

In comparison with other drug classes such as the broad-spectrum kinase inhibitor PKC412 investigated in our laboratory (34), HU210 treatment as single agent seems less efficient in tumor growth reduction. Nevertheless, potential use of cannabinoids as therapeutic intervention for tposRMS should still be pursued, possibly in combination with conventional chemotherapies, kinase inhibitors, or other targeted agents. Several reports indicate synergistic activity of cannabinoid receptor agonists in combination with well-established antineoplastic substances. THC was reported to act synergistically with suboptimal doses of doxorubicin or cisplatin (19), and synergism between HU210 and 5-fluorouracil was recently reported as well (27).

In summary, our results support and extend the previously shown antitumor activities of cannabinoid receptor agonists by showing proapoptotic effects of HU210, THC, and Met-F-AEA on tposRMS cells *in vitro* and, for

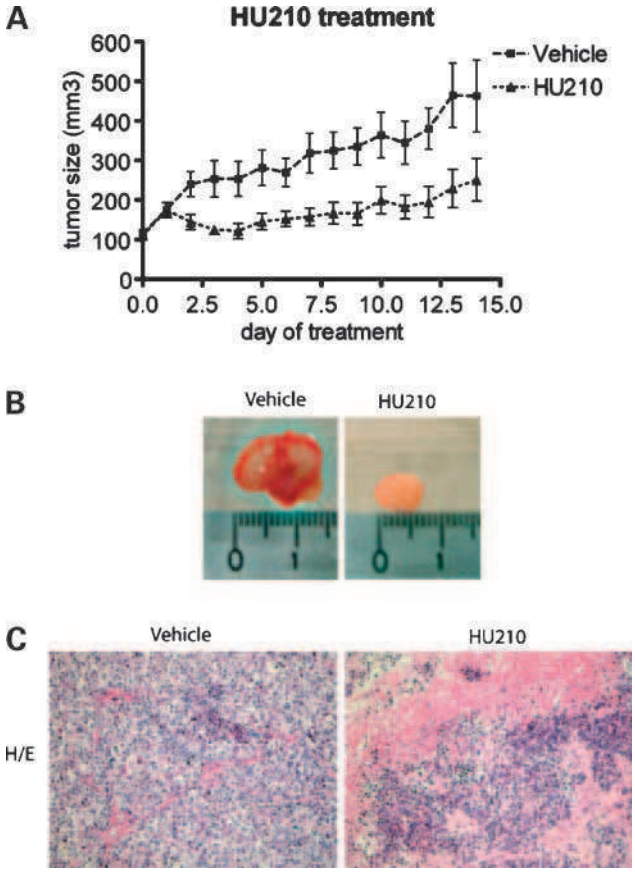

Figure 6. HU210 reduces tumor growth *in vivo*. NOG mice were injected with 7.5×10^6 tposRMS (Rh4) cells s.c. into the flank. After reaching a tumor size of 100 to 150 mm^3, animals were assigned randomly to either the vehicle ($n = 7$) or the HU210 ($n = 6$) group. Treatment was given daily by injecting either 0.2 mg/kg HU210 or DMSO in PBS peritumorally for 13 d, whereas tumor growth was monitored daily and mice were sacrificed on the day after the last treatment. **A,** tumor growth over time is shown for HU210-treated compared with vehicle-treated animals (±SE, $P < 0.001$). **B** and **C,** representative sections of tumors from vehicle- and HU210-treated animals were stained with H&E. Original magnification, 100×.

the first time, show that HU210 has tumor growth inhibiting properties *in vivo*. This could represent one possible novel treatment strategy that might improve outcome in this pediatric tumor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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7. Discussion and Outlook

In the last three decades, it has been reported that not only leukemia (29; 30) and carcinomas (36) are hierarchical organized, also sarcomas such as Ewing's sarcoma have a subpopulation with self renewal properties (100) and are following the cancer stem cell hypothesis (16; 34; 35; 218-221). This so called CSCs share common characteristics with normal stem cells like the potential to self renew and to differentiate into multiple lineages and extensive proliferation rates (27). Due to the better understanding how tumors are organized, new treatment approaches like directly targeting the CSC population are now possible (44; 45; 222). There are reports that RMS follows the cancer stem cell hypothesis, but it is not clear yet and remains to be elucidated which model describes RMS (167; 178; 187; 223; 224). The clonal evolution model is based on the fact that malignant cell clones are expanded in tumors. Contrary, the CSC model argues that a stem-like cell is responsible for tumor development and gives rise to a heterogeneous tumor phenotype (220). Therefore, we investigated, if rhabdomyosarcoma tumors are hierarchically organized and what are the functional characteristics of a CSC population in RMS.

For such a study, we investigated first, whether a subpopulation can be enriched and if this subpopulation is more tumorigenic. Subsequently, we characterized this subpopulation by looking at the differentiation potential, chemoresistance and which markers are specific expressed on this population. Overall, our results demonstrate that eRMS tumors follow the CSC hypothesis. Moreover, the cancer stem cells are more tumorigenic, chemoresistance and represent a RMS tumor phenotype.

7.1 The potential to self renew is one hallmark of cancer stem cells

We first adopted an assay for maintaining and enriching a subpopulation with stem cell properties *in vitro*. Testing different conditions of growth factor concentrations and media, sphere formation could be observed only in one condition which was described previously as a neuronal stem cell medium (225). Furthermore, our data indicated that a subpopulation with sphere forming capability could be enrich over several passages in three eRMS (Ruch2, RD and Rh36) and aRMS (Rh28, Rh30 and RMS13) cell lines. To investigate the tumorigenic potential of Rhabdospheres versus adherent cells, limited dilutions were performed *in vivo*. According to our hypothesis, eRMS spheres were more tumorigenic and fewer cells were needed for tumor development. However, tumor growth was only measured in the highest injection number of aRMS spheres (10^6), whereas 1000 cells were enough for tumor devel-

opment in the parental cell lines leading to the hypothesis that embryonal rhabdospheres are enriched in CSCs, whereas aRMS spheres are non-tumorigenic and might follow the clonal evolution model. Both results favoring the facts that first aRMS and eRMS tumors are not histologically different RMS subtypes, they could be individual tumor types or second aRMS tumors are the more aggressive tumor subtype and thus already enriched in CSCs. To further study the characteristics of the CSC population in rhabdospheres, we focused on the eRMS subtype. Previous studies have shown, that the expression of stem cell genes such as sox2, oct4, c-myc and klf4 being transcription factors and being critical involved with self renewal of undifferentiated embryonic stem cells, can dedifferentiate fibroblasts into induced pluripotent stem cells (iPS). Furthermore, nanog which plays a key role in maintaining pluripotency can function with sox2 and oct4 and establishes an embryonic stem cell identity (226-231). In accordance with these observations, we looked at the expression level of such SC genes of three different rhabdosphere passages (3, 5, 10) and compared it with adherent cells. Additionally, we examined the pax3 expression, because it is upregulated in eRMS patients and plays a crucial role in neuronal and muscle development (181; 182; 196). All SC genes were significantly upregulated in rhabdospheres suggesting that eRMS consist of a subpopulation being enriched in rhabdospheres with self renewal property.

7.2 Differentiation potential of rhabdospheres

The second hallmark of CSCs is the potential to differentiate into multiple lineages and thus give rise to a heterogeneous tumor phenotype. It has been shown that cells with multilineage differentiation potential can differentiate into adipocytes, myocytes and neuronal cells after appropriate stimuli (232-236). On that basis, we next assessed the differentiation potential of rhabdospheres. Therefore we treated rhabdospheres with DMSO and different concentrations of RA. Moreover, we expected the rhabdospheres should differentiate into myocytes, because it was thought that the origin of RMS is mesenchymal (212; 223; 237-240). Additionally, a neuronal differentiation is as well possible due to the high expression of pax3 which is important in neuronal development (181).

Our result demonstrates that rhabdospheres give rise to myogenic and neuronal cells and can differentiate into adipocytes, whereas the adherent cells were negative for the differentiation markers confirming our hypothesis that rhabdospheres have a stem-like phenotype.

7.3 Identification of the CSC population

To date, eRMS cells are enriched in a self renewal subpopulation, but which markers are specifically characterizing this CSC population remains to be elucidated. We chose two different methods which could answer this question, cell surface capturing by mass spectrometry and gene expression profiling.

Cell surface proteins can be identified using mass spectrometry, whereas these proteins should consist of N-glycosylations sites being no longer than 30 amino acids. An advantage of this method is that directly peptide sequences were detected and 2 different cell types can be simultaneously analyzed by labeling the cells with heavy (deuterium) and normal hydrogen. Nevertheless proteins which have longer than 30 amino acids N-glycosylation sites can not be detected and huge number cells are needed (> $8*10^8$ cells) (241). Furthermore, we could not label our rhabdospheres, because they died in that special labeled medium. Therefore, we decided using microarray to investigate the expression profile of eRMS cells and spheres. First, we chose different passages of rhabdospheres (low, middle and high passage) and compared the expression profiles with the parental cell lines to investigate if a signature change is happening. In this regard, more than 2000 genes were up- and downregulated in the sphere cultures. To diminish the amount of genes and to investigate which cell could be the origin of RMS stem cell (178; 179; 239), we performed a meta analysis with several available probesets such as hematopoietic, MSCs, embryonic SCs and neuronal samples. Surprisingly, our eRMS cells and spheres did not correlate with mesenchymal SC and hematopoietic ones, but they clustered with neuronal and glioma cells, spheres and biopsies which could be due to high expression of neuronal genes (184) and pax3 overexpression in rhabdospheres which plays a crucial role in neurogenesis (181). Therefore, the origin of rhabdomyosarcoma stem cells might not be a mesenchymal stem cell. It could be a neuronal precursor which differentiates into a malignant muscle cell after activation (186). In this regard, we looked at the genes commonly up- or downregulated in eRMS and glioma samples. 32 genes, thereof 8 genes encode membrane proteins were commonly upregulated and 12 genes (6 genes encode membrane proteins) downregulated. CD133, a known SC and CSC (38; 44; 45; 56; 57; 61; 98; 111; 177; 242; 243) marker, was one candidate marker, therefore we validated CD133 on protein level. Interestingly, CD133 was as well described in other sarcomas such as Ewing's sarcomas (100) and osteosarcoma (244) suggesting that CD133 could be a sarcoma stem cell marker.

7.4 CD133$^+$ cells favor tumor growth in vivo and are more resistant to chemotherapy

After investigation of CD133 expression on RNA and protein level, we characterized the CD133 positive and negative by performing limited dilutions *in vivo* and clonogenic assays to test the resistance to chemotherapeutics.

After sorting, limited dilutions were performed by orthotopically injecting the different subpopulations into NOD/Scid mice. Tumor growth was readily detected in the control and bulk cells. However, less frequent tumor growth was detected in the sorted population. This could be due to less viable cells after sorting which we also observed *in vitro* experiments. Nevertheless, in the CD133$^+$ injected group one mouse at every dilution tested developed a RMS tumor which was not the case for the CD133$^-$ population suggesting that CD133$^+$ eRMS cells favor tumor growth *in vivo*. Furthermore, in various reports, it has been reported that CSCs are more resistant to chemotherapy and by directly targeting this subpopulation, the tumor loose resistance to chemotherapy (21; 22; 37; 44; 66; 67; 69; 70; 87; 177; 245-247). Therefore, we seeded the sorted cells at low density and treated them with cisPlatin and Chlorambucil. Our data indicate that CD133$^+$ cells are more resistant to chemotherapeutics compared to the negative population and could be a subpopulation responsible for recurrence.

Overall, our results suggest that CD133 is expressed on CSCs in eRMS and thus favor tumor growth *in vivo* and chemoresistance *in vitro*.

7.5 CD133 describes a subpopulation of eRMS patients with poor overall survival

After establishing the CD133, we wanted to evaluate, whether CD133 can be a prognostic marker for eRMS patients. In this regard, we stained a human RMS tissue microarray and analyzed the staining with two variables which we added in the end, intensity of staining (low, middle, high) and number of positive cells. We ended up with four scorings, negative, low, middle and high CD133 expression. The overall survival of eRMS patients is around 70 to 80%. However, if metastasis are detected or relapse occur, survival rates decrease down to 30% (162; 163; 185; 192; 197; 248-251). Our data indicate that patients having high expression of CD133 have a poor overall survival. We could not correlate it to relapse because only 21 patients out of 70 are scored as high positive and 11 high positive patients had a relapse, whereas 7 not and for three patients, the data was missing. Therefore, we could hypothesize

that eRMS patients being highly positive for CD133 favor relapse. In conclusion, we confirmed our CD133 data and after further investigation of more eRMS patients, CD133 might be a prognostic marker in eRMS patients.

7.6 LGR5 another marker upregulated in rhabdospheres

LGR5 also known as GPR49 is a glycoprotein-coupled receptor with leucine-rich repeats extracellular. Since a few years, LGR5 is a known SC marker describing the stem cells of hair follicles (105) and the crypt cells of the intestine (104; 106; 108). Furthermore, it was described as a cancer stem cell marker in several tumors (109; 111; 112; 159). Due to the fact that we found LGR5 upregulation when we compared glioma and Rh36 cells and spheres gene expression profile, we investigated the LGR5 expression on eRMS cells and spheres by Real-time PCR and indeed LGR5 was significantly upregulated in RD and Rh36 spheres. Hence, we stained eRMS cells for LGR5 and CD133. Both markers are expressed on one cell, thus we could hypothesize that LGR5 and CD133 are characterizing the CSC population in eRMS. However, we could not perform FACS analysis, because there is no antibody available recognizing a peptide at the extracellular domain of LGR5. LGR5 was as well described as a target of active Wnt signaling (18; 160; 252) being active when Wnt binds to Frizzled receptor which was also upregulated in rhabdospheres and thus ß-catenin can translocate to the nucleus (152; 253; 254) and activate transcription of target genes. In this regard, we stained rhabdospheres and eRMS cells for ß-catenin to investigate whether Wnt is active. However, we could not detect a nuclei staining. ß-catenin, staining was only detectable in the cytoplasm suggesting the Wnt pathway has to be activated through another mechanism. It was described that Wnt signaling can be activated not through ß-catenin, but through calcium ions. This pathway was then called non-canonical Wnt pathway (155).

Furthermore, it was recently described in basal cell carcinoma (BCC) that LGR5 is a target of the hedgehog pathway (112). BCCs have mutations in the Patched receptor leading to a constitutively active pathway and thus to increased proliferation rates (113). In the late nineties Hahn et al. developed a mouse model with a heterogeneous Patched mutation. These mice developed BCC, RMS and medulloblastoma; because it was shown that all three tumor types have the gorlin syndrome which is characterized by a heterogeneous mutation in the Patched receptor (114-116). However, only 10% of the mice developed RMS. Therefore, for studying the hedgehog pathway in RMS a mouse model with higher RMS incidences is needed. Mao et al. established such a mouse model where smoothened is mutated leading to hedgehog pathway activation. RMS tumors were detected after tamoxifen treatment (117).

Nevertheless, whether LGR5 is a target of the non-canonical Wnt pathway or the hedgehog pathway remains to be elucidated. Therefore, studying RMS in a mouse model combining both LGR5 expression and an active hedgehog pathway would be a great benefit for studying CSCs in eRMS.

7.7 Cell culture versus primary material

Due to the reasons that cancer biology is a complex field of research and there are not enough primary samples in every case, tumor cell lines were established to study cancer biology. Furthermore, cell lines are less complex, totipotent and easy to maintain and enrich in vitro. Therefore, tumor cell lines can be used for example to study the mechanism leading to chemoresistance or to investigate new targeted therapies. However, it might be possible that these cell lines are not characterizing a tumor in general and cell culture results could not be validated on primary material in some cases, for example in glioblastoma (57). This fact raises the question, whether cell culture results are enough to describe specific tumor features, thus primary material is needed to validate the cell culture results.

Every year around 220 new tumor cases under the age of 20 years were diagnosed in Switzerland, whereas only around 15 new RMS cases were detected (~ 5 aRMS and ~ 10eRMS). Furthermore, RMS patient are diagnosed by a spindle biopsy which is followed by chemotherapy and surgery (255). Therefore, the use of primary material in RMS is limited. In addition, all RMS samples were directly frozen or embedded in paraffin for further analysis. Moreover, establishing a protocol for studying fresh primary material might be as well difficult because there is no shared RMS cohort in Switzerland and all tumor samples are directly frozen or embedded in paraffin after a spindle biopsy or surgery. Therefore, the only way to validate RMS cell culture results on primary material is by using a human TMA.

In our case, we could show that CD133 not only describes a subpopulation in cell culture, it also identified a subgroup of eRMS patients having a poor overall survival. Nevertheless, it is necessary to establish a human RMS tumor bank in Europe or at least in Switzerland which can be expanded in immunocompromised mice, thus primary samples can be used for marker validation by injection of different subpopulation into xenografts.

7.8 Summary

In summary, the project of this thesis could show that eRMS cells consist of a subpopulation with self renewal potential. This subpopulation is characterized by the expression of CD133 and the upregulation of several stem cell genes such as oct4, nanog, c-Myc, sox2 and pax3. CD133 could be also correlated with human rhabdomyosarcoma biopsies, where high expression of CD133 was correlated with poor survival. Therefore, CD133 seems a promising marker for identifying a subgroup of eRMS patients in the clinics and could donate new insides in the development of RMS and specific treatment approaches targeting the RMS stem cells can now be envisaged.

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- "Rhabdospheres enrich a CD133 positive potential Rhabdomyosarcoma stem cell population", submitted to PlosONE
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- „CB1 is a potential drug target for treatment of translocation-positive rhabdomyosarcoma", published in the Journal of Molecular Cancer Therapeutics
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- Kolloquium in angewandter Krebsforschung 2010
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